EFFECTS OF CYTOKININ AND CATIONS ON GREENING PROCESS IN EXCISED CUCUMBER COTYLEDONS

by

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A thesis submitted in partial fulfillment of the requirement for the degree of Doctor of Philosophy

May, 1994

Division of Biology

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Abstract

In the present study, the involvements, regulatory effects and sites of action of benzyladenine (BA) and certain cations on greening process were systematically examined. With refer to the greening bioassay in excised cucumber cotyledons, K^+ was demonstrated to stimulate Chlorophyll (Chl) accumulation with an apparent threshold level for maximum stimulation. Na^+ also showed stimulation effect on Chl accumulation but inhibition by 50 mM Na^+ was shown as well. Ca^{2+} was demonstrated to stimulate Chl accumulation but the degree of stimulation was dependent on the amount of Ca^{2+} administrated. Low concentrations of Ca^{2+} (at uM level) and shortened pretreatment period showed pronounced stimulation effect on Chl accumulation. Preventing the changes in cytoplasmic Ca^{2+} by Na_2EGTA and verapamil inhibited Chl accumulation. In addition, inhibition of Chl accumulation by trifluoperazine (TFP) suggests that Ca^{2+} probably regulates Chl formation through a calmodulin-dependent mechanism. It is proposed that Ca^{2+} may act as intracellular messenger of light signal and regulates Chl accumulation through a calmodulin-dependent protein phosphorylation mechanism. For K^+ and Na^+, they are proposed to be involved in facilitating transport of ions into and out of cytoplasm and thereby regulate Chl accumulation.

Ca^{2+} has been suggested to be involved in BA-induced stimulation of Chl accumulation in excised cucumber cotyledons and probably through a calmodulin dependent mechanism. Since treatment of BA-treated cotyledons with Na_2EGTA, verapamil and TFP abolished the BA stimulation effect. In addition, the verapamil inhibition effect was shown to be reversed by the BA treatment in sequence experiment. It is supposed that there existed the antagonism between BA and verapamil: BA may induce an increase in cytoplasmic Ca^{2+} whereas verapamil inhibits it. Ca^{2+} is also proposed as the intracellular messenger of cytokinin signal.

In 5-aminolevulinic acid (ALA) accumulation experiments, the regulatory effects of BA and the cations were also manifested in excised cotyledons but they were not shown in isolated developing chloroplasts. It appears that BA and the cations regulate Chl accumulation at the ALA formation level and their subcellular site of action is probably not in chloroplasts. In contrast, the regulatory effects of BA and the cations may involve nuclear and cytoplasmic events and probably through a Ca^{2+}/calmodulin dependent protein phosphorylation mechanism.

Acknowlegments

I must express my sincerely gratitude to Dr. Y.S. Wong for his patience, guidance and financial support during the past few years. Moreover, I wish to thank Prof. S.T. Chang and Dr. K.H. Yung who sat in the thesis committee and gave me suggestion and criticism. I would also like to thank Prof. S.M. Sun for serving as my thesis external examiner.

My thanks are also due to Mr. Y.H. Wong, Mr. S.C. Lo, Mr. K.S. Cheung, Mr. W.L. Tsang, Ms. P.Y. Cheung, Ms. P.H. Fung, Ms. C.H. Lee, Ms. F.Y. Ng, Ms. P.K. Lee, Ms. S.C. Chan and Ms. B.S. Ko for their support and encouragement throughout my study.

In addition, I would like to thank the members of Team A, Mr. C.O. Tang, Mr. W.L. Chan and Ms. Y.C. Lee for their technical support in my experiments.

Finally, I am grateful to my brothers and sisters in Christ, whose care, love and sharing to make this thesis possible.

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ALA	5-Aminolevulinic acid
ATP	Adenosine 5'-triphosphate
BA	Benzyladenine
BSA	Bovine serum albumin
Chl	Chlorophyll
Chl *a*	Chlorophyll *a*
Chl *b*	Chlorophyll *b*
Chlide	Chlorophyllide
Cys	Cysteine
DMAB	p-Dimethylaminobenzaldehyde
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(β-aminoethylether)N,N,N',N'-tetraacetic acid
Glu	Glutamic acid
Glu-tRNA	Glutamyl-transfer RNA
GSA	Glutamate-1-semialdehyde
GSA-AM	Glutamate-1-semialdehyde 2,1-aminomutase
GluTR	Glu-tRNA reductase
Hepes	(N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
LA	Levulinic acid
LHCP	Light harvesting chlorophyll a/b binding proteins
MgDVP	Mg-2,4-divinyl-pheoporphyrin a_5
Mg-Proto	Mg-protoporphyrin IX

Mg-Proto Me	Mg-protoporphyrin IX monomethyl ester
NADPH	Nicotinamide adenine dinucleotide phosphate, reduced form
PBG	Porphobilinogen
Pchlide	Protochlorophyllide
Proto	Protoporphyrin IX
Protogen	Protoporphyrinogen IX
Tes	(N-tris[Hydroxymethyl]-methyl-2-aminoethane -sulfonic acid)
TFP	Trifluoperazine
$tRNA^{Glu}$	Glutamate transfer RNA

Chapter 1

General Introduction

Greening in etiolated plants is a conspicuous phenomenon concerning the initiation of chlorophyll (Chl) and its protein synthesis in response to illumination (50). As now, light is known to regulate Chl biosynthesis at the level of 5-aminolevulinic acid (ALA) formation (10, 34). Preillumination of etiolated plant materials with a pulse of red light triggers the formation of ALA synthesizing enzymes and thereby eliminates the lag in Chl accumulation after exposure to continuous illumination (10, 16, 37, 38 & 43). Besides, the reduction of protochlorophyllide (Pchlide) to chlorophyllide (Chlide) is also light dependent (1, 10 & 59). The expression of genes for NADPH-protochlorophyllide oxidoreductase is inversely controlled by phytochrome (3, 22). In addition, the enzyme activity and content of enzyme protein decline rapidly upon illumination of etiolated plant materials (22, 25).

Cytokinin and cations are also known to play important role in regulation of greening process (2, 7, 10, 13, 14, 15, 16, 42 & 64). Cytokinin stimulates Chl accumulation with lag phase elimination and steady-state rate acceleration of Chl formation (13, 14 & 16). It is proposed that cytokinin rapidly and specifically initiate the

synthesis of ALA synthesizing enzymes and mimicking the light effect on ALA formation (42). In fact, cytokinin is demonstrated to stimulate ALA formation in the dark (42). Light and cytokinin are shown to have similar effect on ALA and Chl accumulation (7, 10, 13, 16 & 38). For the cations, K^+ and Ca^{2+} have noticeable effects on Chl accumulation (2, 64). K^+ is reported to stimulate Chl accumulation without lag phase elimination and the stimulation effect is light-dependent (2). Ca^{2+} is shown to inhibit Chl accumulation in the early phase of greening by inhibiting ALA formation in the light and accelerating the decomposition of newly formed Chl (64). However, Ca^{2+} is also reported to induce the formation of Chl *b* and light harvesting chlorophyll *a/b* binding proteins (LHCP) in cucumber cotyledons in the dark (65, 66), and also retard Chl degradation in dark-induced senescent plant materials (32, 33 & c.f.64).

As now, greening is known to be light-dependent and is regulated by cytokinin and some cations (2, 10, 13, 42 & 64). However, we know little about the exact molecular mechanisms of light and cytokinin actions. The initial triggering action of the light receptor (presumbly phytochrome) and also the hormone signal perception and transduction processes remain to be determined. In addition, the information concerning the inter-relationship between the effects of light, cytokinin and cations on

greening process is very limited. As mentioned before, light and cytokinin have similar stimulation effect on Chl accumulation (7, 13, 16 & 38) and it is proposed that light and cytokinin may act via the same intermediate steps and share with the same intracellular messenger (presumbly Ca^{2+}) of signal transduction pathways. For cytokinin and cations, Arnold and Fletcher reported that the cytokinin-induced stimulation of Chl accumulation was more pronounced in the presence of K^{+} (2). Moreover, Ca^{2+} is inferred to be involved in certain phytochrome-mediated processes and cytokinin regulated responses in plant cells (5, 12, 60 & 62). Therefore, it is tempting to propose that cations, especially calcium may act as intracellular messenger of light and cytokinin signals (5, 12 & 26).

In the present study, greening as a phytochrome-mediated and cytokinin regulated process in excised cucumber cotyledons is employed to investigate the involvements, regulatory effects and sites of action of benzyladenine (BA, a synthetic cytokinin) and certain cations on Chl biosynthesis. In addition, the possible modes of action of BA and the cations are also proposed.

Chapter 2

Literature Review : Greening Process in Higher Plants

Greening in etiolated plants is a conspicuous phenomenon concerning the initiation of Chl and its binding proteins synthesis in response to illumination (50). It is known that greening is a complicated biochemical process that required the coordinated production of Chl-proteins which are encoded by nuclear and chloroplast genes (18, 50, 51 & 68) as well as the biosynthesis of Chl (4, 10 & 37), and finally assembly of the Chl-protein complexes into the thylakoid membrane in response to cellular or environmental signals (10, 18, 37, 50, 51, 67 & 68).

The Chl biosynthetic pathway is illustrated in figure 2.1. The key precursor of Chl, 5-aminolevulinic acid (ALA) is synthesized from glutamate of Glu-tRNA via the two steps five carbon pathway (34, 35). The first step is the reduction of the activated glutamate to glutamate-1-semialdehyde (GSA) and the second is the transamination of GSA to yield ALA (34, 35). ALA is the building units of tetrapyrrole nucleus (4, 10). Through a sequence of condensation, decarboxylation and oxidation reactions, ALA is converted to protoporphyrin IX (Proto). Proto is the common precursor to heme, bilins and Chl. The insertion of magnesium into Proto begins the Chl branch of the pathway.

Through esterification and isocyclic ring formation, Mg-Proto is converted to protochlorophyllide (Pchlide). And then, Pchlide is reduced to chlorophyllide *a* (chlide *a*) by a light-dependent and NADPH-dependent reaction (1, 4, 10 & 59). However, the reduction reaction is reported to be light-independent in gymnosperms (10). The final step of Chl *a* formation is the addition of long chain polyisoprene phytyl moiety to Chlide *a*. Chl *b* is derived from Chl *a* through a methyl-formyl group conversion (4, 10).

For Chl-proteins, they are the light harvesting Chl *a/b* binding proteins (LHCP) and Chl-protein complexes (CPs) of photosystem I and II (PSI and PSII) (68). LHCP is encoded by nuclear cab gene and CPs of PSI and PSII are chloroplast gene products (50, 68). The formation of Chl-proteins during greening process in cucumber cotyledons is studied extensively by Tanaka's group (65, 66 & 67). LHCP is the main Chl-proteins accumulated in the early phase of greening. As greening proceeded, CPs of PSI and PSII accumulated (67). Indeed, coordinated synthesis and assembly of the Chl-proteins is accomplished in part through coactivation of nuclear and chloroplast gene transcription by cellular or environmental signals (18, 21, 27, 50 & 53). Chl-proteins accumulation is also regulated at translational and/or posttranslational levels (50). Light is known to play an important role on Chl-proteins

synthesis and stability (50, 67). The expression of *cab* gene is photo-regulated at transcriptional level (3, 27). The red light induced accumulation of mRNA level of *cab* gene in etiolated barley is well documented (3). For CPs of PSI and PSII, mRNA levels of chloroplast genes are present in both etiolated and light grown plants, but the apoproteins are only detectable in light grown plants (50). Since the stability of Chl-proteins is dependent on the binding of the apoproteins to Chl molecules (18, 50 & 51). The light-dependent Chl synthesis appears to be the light regulatory mechanism of CPs formation. Consequently, the synthesis of LHCP is photo-regulated at transcriptional level (3, 27) and for the synthesis of CPs of PSI and PSII, it appears to be photo-regulated at translational and/or posttranslational levels (18, 50 & 51).

Chl formation is also photo-regulated (10, 37). The first detectable light-dependent step towards the formation of chloroplast from etioplast is the photo-reduction of Pchlide to Chlide (1, 10 & 59). The photo-reduction reaction is catalyzed by the enzyme NADPH-Pchlide oxidoreductase and is NADPH- and light-dependent (1, 3, 10, 22, 25 & 29). However, the enzyme activity and content of enzyme protein decreased rapidly upon illumination of dark grown plants (22, 25). Häuser *et al.* reported that the inactivation of NADPH-Pchlide oxidoreductase is influenced

by the absence of Pchlide and NADPH (25). The photoconversion of Pchlide and consumption of NADPH for the photo-reduction reaction leads to the proteolytic degradation of the enzyme. In addition, the expression of the genes for NADPH-Pchlide oxidoreductase is inversely controlled by phytochrome at transcriptional level (3, 22). Consequently, it is suggested that the function of the enzyme may be restricted to the early phase of greening. As greening proceeds, the conversion of Pchlide to Chlide may be brought about by another biosynthetic mechanism (10). The ALA formation is another (the first) light-regulatory step in Chl biosynthetic pathway (10, 34). ALA is derived from glutamate via the tRNA-dependent five carbon pathway and the biosynthetic process is catalyzed by glutamyl-tRNA reductase (GluTR) and GSA-2,1-aminomutase (GSA-AM) (34, 35). The enzyme activity is shown to be light-dependent in *Euglena gracilis* (46, 47). In higher plants, molecular studies on the light regulation of Chl biosynthesis in *Arabidopsis* reveals that light may regulate ALA and thereby Chl formation by exerting coordinated transcriptional control over the enzymes of the five carbon pathway (34). Indeed, ALA formation is the rate-limiting step in etiolated plants (10, 42). When etiolated seedlings are first illuminated, the initial photo-reduction of Pchlide to chlide is followed by a lag phase during which the

enzyme system responsible for ALA synthesis appears to be formed *de novo* (10, 42). Chl synthesis only begins in earnest after completion of the lag phase. However, the lag phase can be eliminated by preillumination of the etiolated plant materials with white light or a pulse of red light (10, 16, 37, 38 & 43). It is believed that preillumination-triggered formation in the dark of the ALA synthesizing system, which can then become active immediately upon reillumination (10). In mustard cotyledons, Kasemir *et al.* showed that light has a two-fold effect on Chl formation: lag phase elimination and steady-state rate acceleration (38). These two effects are distinguishable by varying the lengths of the periods of continuous far red light treatment or by varying the lengths of the dark periods after a pulse of red light pretreatment (38). The light-induced two-fold effects on Chl formation is also demonstrated in cucumber cotyledons (13, 16).

Besides the light effect, cytokinin is known to stimulate Chl synthesis in some etiolated plant tissues (2, 7, 10, 13, 14, 15, 16, 42 & 64). In excised cucumber cotyledons, Dei demonstrated that treatment of etiolated plant materials with benzyladenine (BA) eliminated the lag phase and accelerated the steady-state rate of Chl formation during subsequent continuous illumination (13, 14 & 15). In addition, the two cytokinin-induced effects are

shown to be separable either by a brief BA treatment followed by various periods of water treatment in darkness; or by various periods of continuous dark BA treatment (13). It is noted that the two-fold effect of cytokinin on Chl formation has characteristics very similar to that of preillumination (13, 16 & 38). Indeed, light and cytokinin have been shown to have similar effects on the development of etiolated seedlings (7, 10, 13, 16, 38 & 48). The substitution of light effects by cytokinin on the promotion of bean leaf expansion and lettuce seeds germination is known (48). Moreover, Uheda and Kuraishi demonstrated that cytokinin activity in excised etiolated cotyledons of squash was increased after the onset of continuous illumination (70). All these evidences suggested that light (phytochrome) effect is mediated by the action of endogeneous cytokinin (10, 13). However, Dei and Tsuji reported that the actions of light and cytokinin on Chl formation are independent of each other (13, 16). Therefore, The hypothesis that light and cytokinin may act via the same intermediate steps and share with the same intracellular messenger of signal transduction pathway needs further studies.

Cations also play regulatory role on greening process (2, 64) . Among the cations, K^+ and Ca^{2+} have noticeable effects on chl formation (64). Arnold and Fletcher reported

that K^+ stimulated Chl formation without lag phase elimination and the stimulation effect appeared to be light-dependent (2). For the Ca^{2+} effects, Tanaka and Tsuji showed that Ca^{2+} inhibited Chl accumulation in the early phase of greening by inhibiting ALA formation and accelerating the decomposition of newly formed Chl (64). However, in the study of Chl *b* and LHCP formation during light/dark transition of etiolated cucumber seedlings, Ca^{2+} has been shown to substitute the light effect and induce the formation of Chl *b* and LHCP in the dark. In addition, Ca^{2+} is reported to retard Chl degradation in dark-induced senescent plant materials (32, 33 & c.f.64). Consequently, the discrepancy effects of Ca^{2+} on Chl formation and accumulation are dependent on the developmental stages of plant materials studied (64).

As now, greening is known to be light-dependent and is regulated by cytokinin and some cations (2, 10, 13, 42 & 64). Light and cytokinin are demonstrated to exert their effects on ALA formation and thereby stimulate Chl accumulation (7, 10, 13, 16, 38 & 42). However, we know little about the exact molecular mechanisms of light and cytokinin actions. The initial triggering action of phytochrome and the hormonal signal perception and transduction processes remains to be determined. In addition, the information concerning the inter-relationship between the effects of light, cytokinin and cations on

greening process is very limited. As mentioned before, light and cytokinin have similar stimulation effect on Chl formation (7, 13, 16 & 38) and it is proposed that light and cytokinin may act via the same intermediate steps and share with the same intracellular messenger of signal transduction pathways. For cytokinin and cations, Arnold and Fletcher reported that the cytokinin-induced stimulation of Chl accumulation was more pronounced in the presence of K^+ (2). Moreover, Ca^{2+} is inferred to be involved in certain phytochrome mediated processes and cytokinin regulated responses in plant cells (5, 12, 60 & 62). Therefore, it is tempting to propose that cations, expecially calcium may act as intracellular messenger of light and cytokinin signals (5, 12 & 26). In fact, there is increasing interest in defining the role of Ca^{2+} and protein kinase regulatory system in signal transduction processes (23, 55, 56, 57 & 71). It is believed that the identification of new molecules such as genes and novel proteins by the powerful plant molecular biology methods, combined with careful biochemical and physiological analyses will be a promise to reveal the elusive pathways that lie between light, cytokinin and greening process.

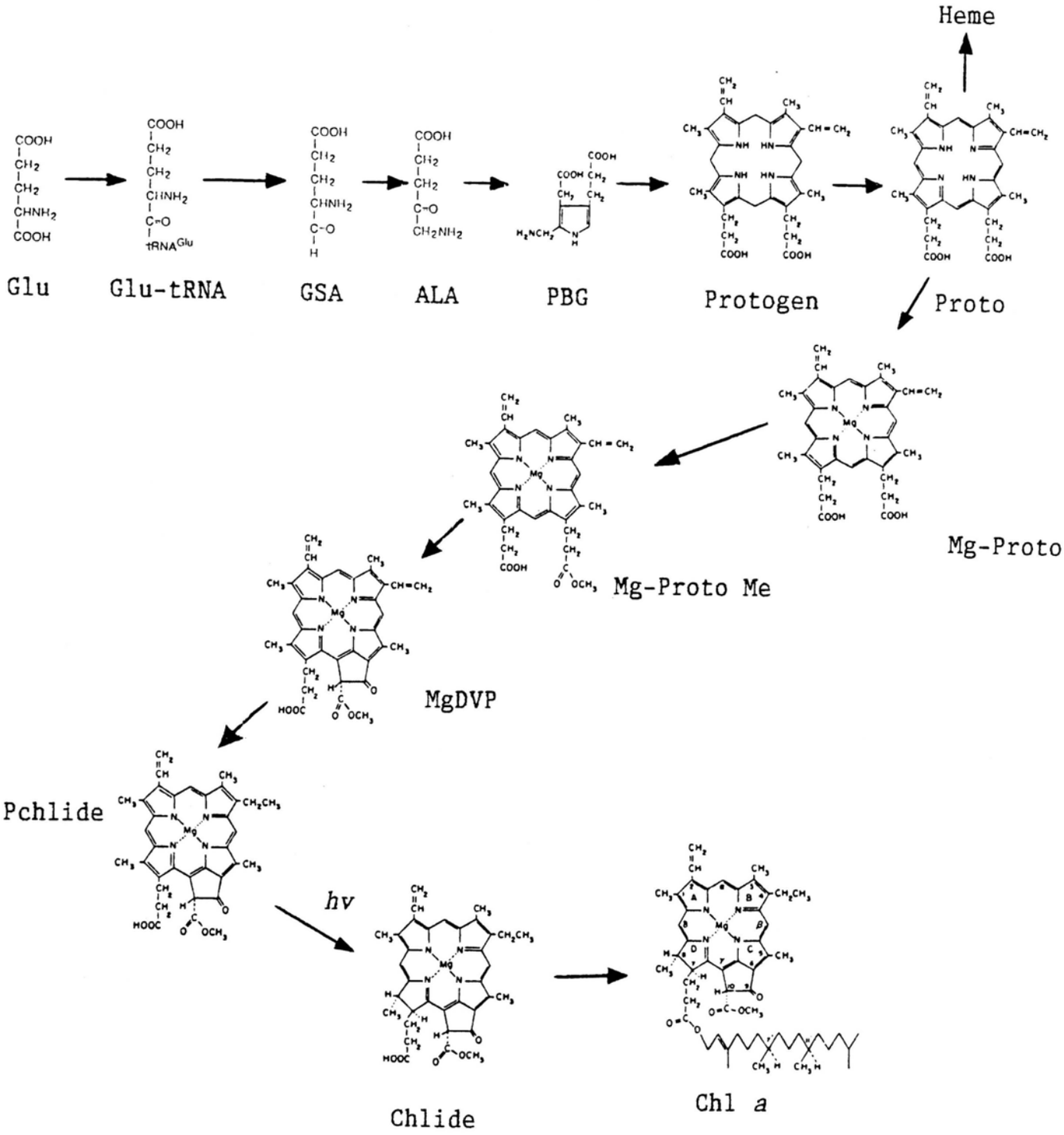

Figure 2.1. The Chl biosynthetic pathway from Glu to Chl *a* with principal intermediates. Abbreviations used: **Glu**, glutamate; **Glu-tRNA**, glutamyl-tRNA; **GSA**, glutamate-1-semialdehyde; **ALA**, 5-aminolevulinic acid; **PBG**, porphobilinogen; **Protogen**, protoporphyrinogen IX; **Proto**, protoporphyrin IX; **Mg-Proto**, Mg-protoporphyrin IX; **Mg-Proto Me**, Mg-protoporphyrin IX monomethyl ester; **MgDVP**, Mg-2,4-divinyl-pheoporphyrin a_5; **Pchlide**, protochlorophyllide; **Chlide**, chlorophyllide; **Chl *a***, chlorophyll *a*.

Chapter 3

Greening Bioassay in Excised Cucumber Cotyledons

Introduction

Greening is a complicated biochemical process that requires the coordinated synthesis of Chl-proteins which are encoded by nuclear and chloroplast genes (18, 50, 51 & 68); as well as Chl biosynthesis (4, 10) and finally assembly of the Chl-protein complexes into thylakoid membrane in response to cellular or environmental signals (10, 18, 37, 50, 51, 67 & 68).

Greening bioassay is the method used for studying the effect of various chemicals and physical factors on Chl synthesis in both excised cotyledons and primary leaves (2, 7, 8, 13, 14, 15, 16, 38, 43, 49 & 64), as well as in intact plants (2, 8, 27, 43 & 58). From early '70, greening bioassay has been frequently employed for various studying purposes such as inter-organ relations in control of greening (49); light effect and hormone effect (2, 7, 8, 13, 14, 15, 16, 38, 43 & 49), as well as the effects of ions (2, 64). Nevertheless, it is known that experimental conditions such as light intensity, greening temperature, age of seedling and oxygen level all have influences on the experimental results obtained and disagreements between these results are known (49). In the present study, the

effects of those experimental conditions on Chl accumulation in excised cucumber cotyledons are examined and try to optimize the experimental conditions for the following experiments.

Materials and Methods

Plant material

Cucumber (*Cucumis sativus* L. cv. Sure Green) seeds were obtained from Known-You Seed Co., Ltd., Taiwan.

Methods

Measurement of Chl synthesizing activity in excised cotyledons of different ages

Cucumber seeds were first immersed in 25 ml distilled water for 15 min before planted in 200 ml vermiculite in plastic flats (18x12.5x8 cm). The seeds were watered once with 250 ml distilled water and were germinated in the dark at 28^{o}C for 4, 5 and 6 days. At the days of harvesting, hypocotyl length was measured and cotyledons were excised into a conical flask containing 25 ml of distilled water under dim green light. Cotyledons were incubated in the dark for 18 h with the flasks kept in orbital shaking at 100 rpm. After 18 h of pretreatment, cotyledons were transferred to petri dishes containing 25 ml of distilled water and exposed to light for 16 h. Table 3.1 shown the relationship between light intensity and temperature of the

light box used in this study. The light box provided 3 conditions of illumination for greening experiments. Condition 1 was used in this experiment. Chl extraction was performed after illumination for 16 h. Ten cotyledons were homogenized in 10 ml of 80% acetone, and then centrifuged at 3000 *g* for 15 min. Chl content in the supernatant was determined by measuring the absorbance at 663 nm and was expressed as ug Chl/cotyledon.

Measurement of Chl accumulation in excised cotyledons after various pretreatment periods

Cucumber seeds were germinated in the dark for 5 days. At the time of harvesting, cotyledons were excised into conical flasks containing 25 ml of distilled water and incubated in darkness for 12 h, 18 h, and 24 h. Afterwards, cotyledons was illuminated for 16 h (condition 1 was used). Chl extraction and determination was performed as previously described.

Measurement of Chl accumulation in excised cotyledons under different light intensities and temperatures

Seed germination, harvesting and pretreatment were carried out as previously described. After pretreatment of the cotyledons for 18 h, cotyledons were brought to illumination for 16 h under various light intensities and temperatures with petri dishes kept in orbital shaking at 50 rpm or stationary. Chl content in the cotyledons was then determined.

Greening curve

Cucumber seeds were germinated in the dark for 5 days and cotyledons were excised into distilled water at the time of harvesting. Cotyledons were incubated in darkness for 18 h, and then exposed to light for various illumination periods. Chl determination was performed as previously described.

Results and Discussion

Effect of seedling age on Chl accumulation

Before initiation of studies on regulation of Chl accumulation, the optimum age of plants should be first determined. The optimum age is determined by consideration of the hypocotyl length distribution and its Chl synthesizing activity, and also the number of seedlings available. In this experiment, the number of cucumber seedlings with their hypocotyl lengths and Chl content of the cotyledons excised from 4-day-old, 5-day-old and 6-day-old plants were determined. Table 3.2a showed the hypocotyl length distribution of etiolated cucumber seedlings of different ages. The hypocotyl length of 4-day-old seedlings was ranged from 4.8 to 7.3 cm and 42% seedlings had an average hypocotyl length of 5.5 cm. For 5-day-old seedlings, hypocotyl length was ranged from 5.8 to 10.9 cm and 79% of seedlings had an average hypocotyl

length of 8.5 cm. The hypocotyl length of 6-day-old seedlings was ranged from 7.5 to 11.5 cm and 63% of seedlings had an average hypocotyl length of 10.1 cm. With correlation of the hypocotyl length to Chl synthesizing activity, table 3.2b showed that maximum Chl synthesizing activity was found in cotyledons excised from 6.1-7.0 cm long 4-day-old seedlings. Chl content in cotyledons excised from 5-day-old plants was almost the same and ranged from 7.2-7.7 ug Chl/cotyledon. Similar result was also found in cotyledons excised from 6-day-old plants but Chl content was ranged from 5.2-5.6 ug Chl/cotyledon. Chl synthesizing activity decreased as age of seedlings increased. By consideration of the availability of seedlings with acceptable Chl synthesizing activity, 4-day-old plants had maximum Chl synthesizing activity but variation was quite large (as reflected from SE). In addition, the availability of 4-day-old seedlings that suited for experiment was very limited (only 42% could be used). In contrast, the availability of the 5-day-old and 6-day-old seedlings with an acceptable Chl synthesizing activity was much higher. The Chl content in 5-day-old plants was higher than that in 6 day-old plants. Consequently, 5-day-old etiolated cucumber seedlings of hypocotyl length ranges from 7.7 to 9.5 cm were used in the following experiments.

Effect of pretreatment period on Chl accumulation

Dark-induced senescence in detached leaves and excised cotyledons is well documented (32, 33). Senescence is resulted in loss of Chl and protein degradation and is regarded as undesirable in greening bioassay. Therefore, the selection of pretreatment period is very important as dark-induced senescence must be prevented. In this experiment, pretreatment periods of 12 h, 18 h and 24 h were tested. Table 3.3 showed the effect of pretreatment time on Chl accumulation. No significant decrease in Chl content was found even pretreatment period prolonged to 24 h. Nevertheless, pretreatment period of 18 h was suggested when cotyledons are excised into test solutions. Since prolonged exposure of excised cotyledons to some chemcials may be undesirable and may obscure their effects on Chl accumulation. Consequently, pretreatment period of 18 h was used in the following experiments.

Effects of light intensity and temperature on Chl accumulation

As shown in table 3.1, light intensity and temperature are two inter-related parameters which cannot change one without changing the other. Temperature control by ventilation is very limited. In this experiment, the effects of light intensity and temperature on Chl accumulation was investigated. Cotyledons illuminated under

light intensities at 50 and 55 $uEm^{-2}s^{-1}$ PAR (temperature is $28^{o}C$ and $29^{o}C$) showed no significant difference in Chl content (Table 3.4). However, when light intensity increased to 60 $uEm^{-2}s^{-1}$ PAR (temperature increased to $31^{o}C$), Chl accumulation decreased. It seems that Chl accumulation was retarded at high temperature. Consequently, light intensity at 50 $uEm^{-2}s^{-1}$ PAR with temperature at $28^{o}C$ was used for the following experiments.

Effect of shaking (for oxygen replenishment) on Chl accumulation

Chl formation is an oxygen-dependent process (10). Unfavourable oxygen levels will result in retardation of chlorophyll formation (49). The replenishment of oxygen to excised cotyledons during pretreatment and illumination was achieved by orbital shaking at 100 rpm and 50 rpm respectively in this study. Table 3.4 showed the effect of shaking on Chl synthesis under various light intensities. Under the light intensities tested, Chl content in cotyledons kept in shaking and stationary were almost the same, except that 30 to 50% cotyledons which kept in stationary during illumination sinked to the bottom of petri dish and Chl content is about 1.4 ug Chl/cotyledon. Unfavourable oxygen levels retarded Chl formation. Therefore, cotyledons were kept in shaking during illumination.

Greening curve

Figure 3.1 showed the greening curve of excised cotyledons of cucumber cultivar Sure Green. At the first 2 h after the onset of illumination, rate of Chl formation was very slow and a lag phase was recognized. Afterwards, rate of Chl formation was accelerated exponentially. Stationary phase was attended after 20 h of illumination. Based on the greening curve, illumination time of 16 h was used for the following experiments.

Greening bioassay in excised cucumber cotyledons

Based on the experimental findings, cucumber seeds were germinated in the dark at 28^{o}C for 5 days. At the day of harvesting, cotyledons were excised into conical flask containing 25 ml of test solutions or distilled water under dim green light. Cotyledons were incubated in darkness for 18 h during pretreatment. Afterwards, cotyledons were transferred to petri dishes containing 25 ml of test solutions or distilled water and were illuminated at 50 $uEm^{-2}s^{-1}$ PAR for 16 h with petri dishes kept in orbital shaking.

Table 3.1. Relationship between light intensity and temperature of the light box used in the present study.

Condition	Light intensity ($uEm^{-2}s^{-1}$)	Temperature with electric fan	
		turned on	turned off
1	50	28°C	30°C
2	55	29°C	32°C
3	60	31°C	35°C

Table 3.2a The distribution of hypocotyl length of etiolated cucumber seedlings of different ages. Cucumber seeds were germinated in the dark at 28°C. At the day of harvesting, hypocotyl length of the seedlings was measured. Number of seedling and mean hypocotyl length±SD was shown in columns.

Age of seedling	number of seedling (and mean hypocotyl length)							
	Length of hypocotyl (cm)							
	4.1-5.0	5.1-6.0	6.1-7.0	7.1-8.0	8.1-9.0	9.1-10.0	10.1-11.0	11.1-12.0
4-day-old	21 (4.8±0.3)	41 (5.5±0.3)	27 (6.4±0.3)	8 (7.3±0.2)	---	---	---	---
5-day-old	---	6 (5.8±0.3)	17 (6.7±0.4)	36 (7.7±0.2)	33 (8.5±0.2)	30 (9.5±0.4)	3 (10.9±0.2)	---
6-day-old	---	---	---	16 (7.5±0.3)	18 (8.6±0.3)	33 (9.5±0.2)	36 (10.6±0.3)	7 (11.5±0.3)

Table 3.2b. Chl accumulation in cucumber cotyledons excised from etiolated plants of different ages. Cotyledons were excised into distilled water and incubated in the dark for 18 h, and then exposed to light (50 uEm-2s-1 PAR) for 16 h. Chl content was expressed as ug Chl/cotyledon and mean values with SE were shown in columns.

Age of seedling	Chl content (ug Chl/ cotyledon)							
	Length of hypocotyl (cm)							
	4.1-5.0	5.1-6.0	6.1-7.0	7.1-8.0	8.1-9.0	9.1-10.0	10.1-11.0	11.1-12.0
4-day-old	8.73±0.22	8.53±0.27	10.35±0.24	9.23±0.37	---	---	---	---
5-day-old	---	7.16±0.12	7.73±0.03	7.38±0.20	7.14±0.20	7.27±0.26	7.08±0.12	---
6-day-old	---	---	---	5.62±0.52	5.66±0.39	5.54±0.32	5.29±0.26	5.24±0.12

Table 3.3. Effect of pretreatment period on the Chl accumulation in cucumber cotyledons excised from five-day-old plants. The cotyledons were dark pretreated with distilled water for various periods and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Mean values with SE were shown in column, and means within column followed by the same letter are not significantly different at P=0.05 according to Student-Newman-Keuls test. Experiment was done twice with 7 to 8 replicates in each treatments.

Pretreatment period	Chl content (ug Chl/cotyledon) after 16 h illumination
12 h	7.37±0.12*a*
18 h	7.49±0.19*a*
24 h	7.57±0.16*a*

Table 3.4. Effects of light intensity and temperature on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with distilled water for 18 h and then exposed to light for 16 h at various light intensities with the cotyledons kept in orbital shaking or stationary. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Mean values with the SE were shown in the columns, and means wuthin columns followed by the same letter are not significantly different at P=0.05 according to Student-Newman-Kuels test. Experiment was repeated twice with 5 to 8 replicates in each treatments.

Light intensity ($uEm^{-2}s^{-1}$ PAR)	Temperature	Chl content (ug Chl/cotyledon) in cotyledons kept in	
		Orbital shaking	Stationaty
50	28°C	7.87±0.12*a*	7.74±0.27*a* (1.48±0.05)
55	29°C	8.17±0.12*a*	6.93±0.08*b* (1.45±0.02)
60	31°C	7.09±0.13*b*	6.81±0.13*b* (1.30±0.08)

Remark : Chl content in the parentheses was the amount of Chl produced by cotyledons kept in stationary during illumination but were sinked to the bottom of petri dish.

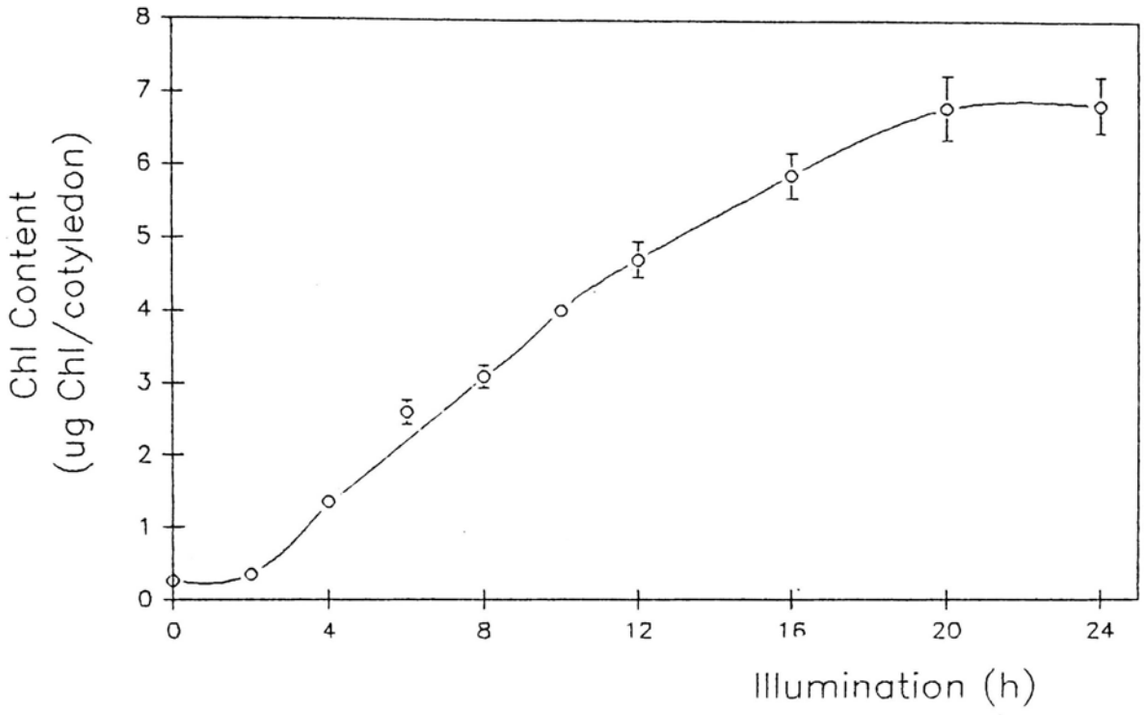

Figure 3.1. Time course of Chl accumulation under continuous illumination in excised cucumber cotyledons. Cotyledons were dark incubated for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for various periods. Chl content of 10 ml of 80% acetone extract of ten cotyledons was expressed as ug Chl/cotyledon. Each point represented the mean of 8 replicate samples.

Chapter 4

Regulation of Chlorophyll Accumulation in Excised Cucumber Cotyledons

Introduction

Cytokinin and cations are known to play important roles in regulation of various levels of cell functions in higher plants (2, 7, 10, 13, 14, 15, 16, 42 & 64). During greening, cytokinin stimulates Chl accumulation and has been shown to have two-fold action on Chl formation (13, 14). Among cations, K^+ and Ca^{2+} have noticeable effects on Chl accumulation (2, 64); K^+ promotes while Ca^{2+} inhibits in the early phase of greening. However, Ca^{2+} also has been shown to induce the formation of Chl *b* and light harvesting Chl *a/b* protein complex in excised cucumber cotyledons in the dark (65, 66), as well as retard Chl degradation in dark-induced senescent plant materials (32, 33 & c.f.64). In contrast to K^+ and Ca^{2+}, the effect of Na^+ on Chl accumulation has not been mentioned.

In the present study, the effects of benzyladenine (BA); Na^+, Ca^{2+} and K^+ on Chl accumulation in excised cucumber cotyledons were examined by different experimental approaches. The possible modes of action of BA and the cations are also proposed.

Materials and Methods

Materials

Cucumber seeds (*Cucumis sativus* L. cv. Sure Green) were obtained from Known You Seed Co., Ltd., Taiwan.

The following were purchased from Sigma: BA, $CaCl_2$, ethylene glycol-bis(β-aminoethylether)N,N,N'N'-tetraacetic acid (EGTA), KCl, verapamil and trifluoperazine (TFP). NaCl was obtained from Anala R; acetone was from Mallinckrodt.

Methods

Seed germination

Cucumber seeds were soaked in distilled water for 15 min before planted in vermiculite which had been watered once with 250 ml distilled water. Seeds were germinated in the dark at 28°C for 5 days.

Harvesting and pretreatment

At the day of harvesting, cotyledons were excised without their hypocotyl hooks and eighty cotyledons were placed into a conical flask containing 25 ml of test solution. All manipulation described above was performed under dim green safelight. Dark pretreatment was carried out at 28°C for 18 h with the conical flasks kept in orbital shaking at 100 rpm. In some experiemnts, changes of test solutions during dark pretreatment were performed.

The test solutions in this study included various concentrations of BA, Na_2EGTA, NaCl, $CaCl_2$, KCl, verapamil and TFP.

Illumination and Chl determination

After 18 h of dark pretreatment, cotyledons were transferred to petri dishes containing 25 ml of test solutions and were illuminated under the light intensity at 50 $uEm^{-2}s^{-1}$ PAR for 16 h. Chl of ten cotyledons was extracted with 10 ml of 80% acetone and the homogenate was centrifuged at 3000 *g* for 15 min. Chl content in the supernatant was determined by measuring absorbance at 663 nm and was expressed as ug Chl/cotyledon.

Results

Effect of BA on Chl accumulation

BA was shown to stimulate Chl accumulation in excised cotyledons of cucumber cultivar Sure Green (Table 4.1). The BA-induced stimulation effect was demonstrated in all of the BA concentrations tested. $5x10^{-8}$ M BA showed 20% increase in Chl content, $5x10^{-6}$ M BA stimulated Chl accumulation by 59%. The BA stimulation effect increased as concentrations of BA increased. However, the BA stimulation effect decreased markedly at $5x10^{-5}$ M and resulted in 32% increase in Chl content. Arnold and Fletcher reported that prolonged exposure of excised cotyledons to BA tended to be

toxic and decreased the stimulation effect on Chl formation (2). Therefore, it seems that treatment of excised cotyledons with high concentration of BA (e.g. $5x10^{-5}$ M in this experiment) or prolonged BA pretreatment will inhibit the BA stimulation effect. $5x10^{-6}$ M BA was shown to be the most effective concentration in stimulation of Chl accumulation.

Besides the concentrations effect, the BA-induced two-fold action on Chl formation was shown in figure 4.1. 5 uM BA stimulated Chl accumulation in excised etiolated cotyledons with lag phase elimination and steady-state rate acceleration after the onset of illumination. Chl content in the BA-treated cotyledons was increased by 34% after 4 h of illumination, 42% after 10 h and 60% after illumination for 14 h and 16.5 h.

Effect of Na_2EGTA on Chl accumulation

Na_2EGTA is a calcium specific chelator used in many systems to alter endogenous Ca^{2+} concentration by causing a depletion of cytoplasmic Ca^{2+} (9). In this experiment, Na_2EGTA of concentrations ranging from 1 mM to 20 mM were tested. Na_2EGTA was shown to inhibit Chl accumulation and the degree of inhibition increased as concentrations of Na_2EGTA increased (Table 4.2). 1 mM Na_2EGTA showed 21% inhibition of Chl accumulation and 4 mM Na_2EGTA showed 46% inhibition. Na_2EGTA at 10 mM or higher inhibited Chl

accumulation by 80%. Chl content was decreased by the Na_2EGTA treatment.

In order to determine whether the Na_2EGTA inhibition effect was only mediated through the chelation of cytoplasmic free calcium, exogeneous calcium (as $CaCl_2$) was applied to Na_2EGTA-treated cotyledons during dark pretreatment. It was assumed that the complex formation between exogeneous Ca^{2+} and EGTA (in a ratio of 1:1) would abolish the Na_2EGTA inhibition effect. From the experimental results, the inhibition effect caused by 1 mM, 2 mM and 4 mM Na_2EGTA were not only abolished by the addition of $CaCl_2$ of corresponding concentrations, but also stimulation of Chl accumulation was shown (Table 4.3). For cotyledons treated with 10 mM $CaCl_2$+Na_2EGTA, Chl accumulation was decreased by 60%. As $CaCl_2$+Na_2EGTA mixture composed of Na^+, Cl^- and CaEGTA complexes which was supposed having no effect on Chl accumulation, the stimulation effect may be contributed by Na^+ and Cl^- ions. Table 4.4 showed the effects of $CaCl_2$+Na_2EGTA and NaCl of concentrations equivalent to the ionic strengths of the $CaCl_2$+Na_2EGTA mixtures on Chl accumulation. 8 mM NaCl stimulated Chl accumulation by 13% as 4 mM $CaCl_2$+Na_2EGTA did. Similar result was also obtained in 1 mM NaCl-treated and 0.5 mM ($CaCl_2$+Na_2EGTA) -treated cotyledons. Therefore, the ($CaCl_2$+Na_2EGTA)-induced stimulation of Chl accumulation

was contributed by Na^+ and Cl^- ions. In another experiment performed with $CaCl_2$+Na_2EGTA with ratios of Ca^{2+} to Na_2EGTA other than 1:1, results were shown in table 4.5. It was shown that the Na_2EGTA inhibition effect can only be abolished in the presence of excess Ca^{2+} ions. The Chl content in cotyledons treated with 5 mM $CaCl_2$ + 4 mM Na_2EGTA was 102% of water control. Stimulation effect was also demonstrated in 4 mM ($CaCl_2$+Na_2EGTA)-treated cotyledons and resulted in 25% increase in Chl content. For cotyledons treated with 2 mM $CaCl_2$ + 4 mM Na_2EGTA and 1 mM $CaCl_2$ + 4 mM Na_2EGTA, the Na_2EGTA inhibition effect was partially reversed by the $CaCl_2$ treatment.

Effects of Na^+, Ca^{2+} and K^+ on Chl accumulation

Cations are known to play important roles in Chl formation. Among cations, K^+ and Ca^{2+} have noticeable effects on Chl accumulation (2, 64) but the effect of Na^+ has not been mentioned. In this study, Na^+ (as NaCl) was demonstrated to stimulate Chl accumulation (Table 4.4) and concentrations effect of NaCl was shown in table 4.6. NaCl of concentrations ranged from 0.5 mM to 10 mM stimulated Chl accumulation. At 0.5 mM and 10 mM, Na^+ increased Chl content by 14%. 1 mM Na^+ showed 20% stimulation of Chl accumulation and was the most effective concentration.

As reported by Tanaka and Tsuji (64), Ca^{2+} inhibited

Chl accumulation in the early phase of greening. Low concentrations of Ca^{2+} (10 mM) slightly promoted Chl accumulation but as concentrations increased Chl content decreased. The effect of Ca^{2+} on Chl accumulation was shown in table 4.7. $CaCl_2$ of concentrations ranged from 1 uM to 10 mM were examined. From the experimental results, $CaCl_2$ ranged from 1 uM to 1 mM slightly increased Chl content and showed 2% to 6% stimulation of Chl accumulation. At 5 mM and 10 mM, Ca^{2+} inhibited Chl accumulation by 20% and 33% respectively. Low concentrations of Ca^{2+} (at uM level) slightly stimulated Chl accumulation but as concentration increased Chl content decreased. It was noticed that the experimental results in the present study was similar to those obtained by Tanaka and Tsuji although the $CaCl_2$ administration method (and thus the concentrations of Ca^{2+} showing the effects) and greening periods were different in the two studies.

K^+ was known to stimulate Chl formation. The concentrations effect of K^+ on Chl accumulation was shown in table 4.8. KCl of concentrations ranged from 1 mM to 20 mM stimulated Chl accumulation by almost 100%. The most effective concentration was 4 mM KCl and resulted in 114% increase in Chl content. In the experiment investigating the ionic strength effects on Chl accumulation, the stimulation effects of Na^+, Ca^{2+} and K^+ of concentrations

ranged from uM to mM levels were compared (Table 4.9). Na^+ was demonstrated to stimulate Chl accumulation by about 20% in all of the concentrations tested. Stimulation by Ca^{2+} of Chl accumulation was more pronounced at uM level. 3.3 uM Ca^{2+} increased Chl accumulation by 16%. K^+ stimulated Chl accumulation but as concentrations decreased stimulation effect decreased. The K^+ stimulation effect was more pronounced at mM level. Therefore, it appears that stimulations of Chl accumulation by Na^+, Ca^{2+} and K^+ were not non-specific ionic strength effect but were specific to each cations.

Effects of duration of pretreatment with Na^+, Ca^{2+} and K^+ on Chl accumulation

Table 4.10a showed the effects of treatment of excised cotyledons with 1 mM NaCl, 10 uM $CaCl_2$ and 4 mM KCl respectively for different time intervals during dark pretreatment and illumination on Chl accumulation. Na^+ stimulated Chl accumulation by 20% and the stimulation effect was independent on the duration of pretreatment periods. Ca^{2+} was demonstrated to increase Chl accumulation but the stimulation effect decreased as pretreatment period prolonged. Cotyledons pretreated with 10 uM $CaCl_2$ for 9 h in darkness resulted in 17% increase in Chl content. When pretreatment periods prolonged to 15 h and 18 h, the Ca^{2+} stimulation effect decreased to 10% and 6% respectively. In

contrast to Ca^{2+}, prolonged pretreatment of excised cotyledons with KCl resulted in increasing stimulation of Chl accumulation. The K^+-induced stimulation effect increased from 54% for 9 h dark pretreatment to 95 % for 18 h dark pretreatment. Both of the stimulation effects of Ca^{2+} and K^+ were dependent on the duration of pretreatment periods. In addition, by comparing the same stimulation effects exerted by the cotyledons treated with cations only during dark pretreatment (18 h) with those treated with cations only during illumination (16 h), it was found that stimulation of Chl accumulation by the cations was only dependent on the exposure time of excised cotyledons to cations regardless of the cations were administrated in the dark or in the light.

Table 4.10b presented the results of comparing the effects of Na^+, Ca^{2+} and K^+ on Chl accumulation in different pretreatment periods. Na^+, Ca^{2+} and K^+ stimulated Chl accumulation in all of the pretreatment periods tested. Among the cations, the K^+ stimulation effect was the most pronounced. Na^+ increased Chl accumulation by 20% regardless of the duration of pretreatment periods. Stimulation of Chl accumulation by Ca^{2+} was more pronounced at short pretreatment period (9 h). As the Ca^{2+} stimulation effect was also more pronounced at low concentration (Table 4.9), it appeared that low concentration of Ca^{2+} was

equivalent to short pretreatment period and both exerted similar effects on Chl accumulation.

Effect of verapamil and Ca ionphore A23187 on Chl accumulation

Changes in cytoplasmic calcium has been inferred to be involved in certain phytochrome-mediated processes and cytokinin regulated responses in plant cells (5, 12, 60 & 62). The presence of Ca^{2+} channels and Ca^{2+}-ATPase on plasma membrane, tonoplasts, endoplasmic reticulum, as well as chloroplasts and mitochondria allowes intracellular Ca^{2+} concentration changes (5, 23). Ca channel blocker verapamil and Ca ionophore A23187 which modulate intracellular Ca^{2+} concentrations are frequently employed for studies of Ca^{2+}-regulated cellular responses in animal and plant cells (23, 62). In this study, verapamil of concentrations ranged from 1 uM to 5 mM were tested. Verapamil inhibited Chl accumulation and the inhibition effect increased with increasing concentrations (Table 4.11). 10 uM verapamil inhibited Chl accumulation by 11%. 100 uM verapamil decreased Chl content by 40%. At 1 mM or higher, verapamil inhibited Chl accumulation by 89%. Blockage of natural increase in cytoplasmic calcium by verapamil treatment inhibited Chl accumulation.

Table 4.12 presented the concentrations effect of ionophore A23187 on Chl accumulation. Experimental generation of an increase in cytoplasmic calcium by

ionophore A23187 inhibited Chl accumulation. The inhibition effect increased as concentrations of ionophore increased. 50 nM A23187 showed 12% inhibition of Chl accumulation. A23187 at 5 uM inhibited Chl accumulation by 70%. At 10 uM or higher, A23187 decreased Chl content by 85%. It seemed that non-regulated increase in cytoplasmic calcium by ionophore A23187 was inhibitory on Chl accumulation.

Effect of TFP on Chl accumulation

In many Ca^{2+}-regulated cellular responses, calcium effects are mediated through calmodulin-dependent mechanism (11, 17, 19, 20, 23, 26, 32, 36, 39, 40, 52, 55, 56, 57, 61 & 71) . Calmodulin has no enzymatic activity of its own, but it modulates the activities of several enzymes (e.g. protein kinases) and some non-enzymic proteins in a calcium-dependent manner (11, 61). The structure, function and homeostasis of calmodulin in plant cells is reviewed by Roberts and Harmon (61). In the present study, the involvement of calmodulin in Chl accumulation was demonstrated by the TFP treatment. TFP is an antipyschotic drug and is known to be a calmodulin-specific antagonist (41). The concentration effects of TFP on Chl accumulation was shown in table 4.13. TFP inhibited Chl accumulation and as concentrations increased Chl content decreased. 10 uM TFP showed no inhibition effect on Chl accumulation. 100 uM

TFP decreased Chl content by 43%. TFP at 200 uM showed 91% inhibition of Chl accumulation.

Discussion

Effect of BA on Chl accumulation

BA is known to stimulate Chl accumulation in etiolated cucumber cotyledons (2, 7, 10, 13, 14, 15, 16 & 42). In the present study, BA-stimulated Chl accumulation in excised cotyledons of cucumber cultivar Sure Green was confirmed. The BA stimulation effect increased as concentrations increased (Table 4.1). However, a marked reduction of BA effect was observed at $5x10^{-5}$ M. As reported by Dei (13), BA appeared to act as a trigger of Chl formation and was metabolized to inactive form very rapidly. It seems unlikely that the stimulation effect would decrease at high BA concentration. However, Arnold and Fletcher reported that prolonged treatment with BA tended to be toxic and decreased the BA stimulation effect (2). It is supposed that prolonged treatment with BA is equivalent to high BA concentration treatment and both decreased the BA effect. Nevertheless, the toxicity of BA remains to be determined. As now, nothing is known regarding the molecular mechanism of BA action and it is believed that only exploration of the molecular mechanism of BA action is a requisite in understanding the BA effects on Chl accumulation.

Besides the concentration effect, BA was shown to have two-fold action on Chl accumulation: lag phase elimination and steady-state rate acceleration of Chl formation (Figure 4.1). However, the BA effect on lag phase elimination was less pronounced as compared with other studies (13). Since the degree of stimulation and pattern of the two-fold action of BA are dependent on the duration of pretreatment period and the concentration of BA administrated (13, 42). Therefore, it is believed that the BA-stimulation pattern in cucumber cultivar Sure Green was unique to the pretreatment period and the BA concentration used in this study.

Effects of Na^+, Ca^{2+} and K^+ on Chl accumulation

Cations are known to play important role in Chl accumulation (2, 64). Among cations, K^+ and Ca^{2+} have noticeable effects on Chl accumulation but the effect of Na^+ has not been mentioned. In the present study, K^+ was demonstrated to stimulate Chl accumulation. The stimulation effect increased as K^+ concentration increased from uM to mM levels, as well as increased with prolonged pretreatment periods. In contrast to K^+, Na^+ stimulated Chl accumulation but was concentration independent. Prolonged pretreatment with Na^+ did not result in further increase in Chl content. Low concentrations of Ca^{2+}, at uM level was demonstrated to stimulate Chl accumulation but as concentration increased

to mM level Chl content decreased. Tanaka and Tsuji reported that Ca^{2+} inhibited Chl accumulation in the early phase of greening was resulted from the inhibition by Ca^{2+} of ALA formation in the light and accelerating the destruction of newly formed Chl (64). It is not known whether the inhibition effect caused by high Ca^{2+} concentrations in this study was resulted from the same reasons as reported by Tanaka and Tsuji. Since the Ca^{2+} administration methods and greening periods were different in the two studies. Moreover, the Ca^{2+} stimulation effect was shown to be more pronounced when dark pretreatment period was shortened to 9 h. Indeed, it is known of no convincing experiments in demonstrating the compartmentizat -ion and penetration of exogeneously applied cations into the cytoplasm as well as the mechanism of action of the cations on cell functions in plant cells. It is proposed that the exogeneously applied cations were accumulated outside the plasma membrane and ion uptake was mediated via ion channels. Phytochrome-mediated Ca^{2+} uptake in maize protoplasts is well documented (12). Taking *Vicic faba* guard cells as an example to illustrate the interactions between ions, ion channels, and thus the regulatory mechanism of ion transport across plasma membrane, Schroeder reported that K^+ uptake was accompanied by blue light activated proton extrusion across plasma membrane.

Elevation of cytosolic free Ca^{2+} inhibited K^+ uptake but stimulated K^+ release (63). All these evidences showed that ion uptake across plasma membrane was a complicated process that involving interactions between ions and ion channels; and also may be photo-regulated.

With refer to the experimental results, low concentration of Ca^{2+} was equivalent to short pretreatment period and both exerted pronounced stimulation effect on Chl accumulation. It seems likely that the amount of Ca^{2+} which accumulated outside plasma membrane and penetrated into cytoplasm was almost the same by the two treatments and hence exerted similar effects on Chl accumulation. Therefore, the Ca^{2+} stimulation effect seems to depend on the amount of Ca^{2+} administrated either adjusted by concentrations or pretreatment periods. For the effect of K^+, stimulation of Chl accumulation by K^+ was relatively constant at mM level (about 100% stimulation), but as duration of pretreatment period decreased stimulation effect decreased. Moreover, the K^+ stimulation effect decreased markedly at uM level. It appears that K^+ had a threshold level for maximum stimulation of Chl accumulation. Administration of K^+ beyond the threshold level did not result in further increase in Chl content. In contrast to Ca^{2+} and K^+, stimulation by Na^+ of Chl accumulation was independent on concentrations and

pretreatment period. The Na^+ stimulation effect seems to possess a very low threshold level for maximum stimulation of Chl accumulation. Consequently, the stimulation effects of Na^+, Ca^{2+} and K^+ are totally different from each others and are unique to each cations.

Taking account of the cytoplasmic concentrations of Na^+, Ca^{2+} and K^+ into the consideration of the mechanism of action of the cations on cell functions, Ca^{2+} has an advantage over the other abundant cations in acting as a carrier of information (messenger) because of its low intracellular concentration (5, 26). Since intracellular $[Ca^{2+}]$ is submicromolar, tranisent increase of cytoplasmic concentration to 1-10 uM will cause a large change in ion concentration but without disturbing the osmotic and charge balance within the cells. In addition, the affinity of calmodulin for Ca^{2+} and response element (eg. protein kinases) that determine the cellular response is dependent on the changes in cytoplasmic calcium concentration, and not on its absolute concentration. In contrast, Na^+ would have to change from 10^{-3} to 10^{-1} and K^+ from 10^{-1} to 10 M to provide an equivalent increase in affinity and thus will seriously disturb the osmotic and charge balance within the cells and would require excessive energy to restore homeostasis. Consequently, it is proposed that Ca^{2+} may act as intracellular messenger of light signal and modulate Chl

formation and accumulation probably through a protein kinase regulatory system. For effects of K^+ and Na^+, K^+ has a higher cytoplasmic concentration than Na^+ and therefore, it is reasonable for K^+ possesses a higher threshold level for maximum stimulation of Chl accumulation than Na^+. It is supposed that Na^+ and K^+ may facilitate transport of ions (especially Ca^{2+}) into and out of cytoplasm and thereby regulate Chl accumulation. Nevertheless, the mechanisms of Na^+- and K^+-induced stimulation of greening remain to be studied.

Demonstration of the involvement of Ca^{2+} in Chl accumulation

Jaffe enunciated three rules for defining the involvement of Ca^{2+} in a cellular response (c.f.26). They are: 1. the response should be preceded or accompanied by an increase in intracellular [Ca^{2+}]; 2. blockage of the natural [Ca^{2+}] increase should inhibit the response; and 3. experimental generation of an increase in the intracellular [Ca^{2+}] should stimulate the response. In the present study, the involvement of Ca^{2+} in Chl accumulation was demonstrated by two different approaches. The first one is the prevention of intracellular [Ca^{2+}] changes through the chelation of cytosolic free Ca^{2+} by Na_2EGTA and blockage of increase in intracellular [Ca^{2+}] by verapamil. The second approach is the experimental generation of an increase in

intracellular [Ca^{2+}] by ionophore A23187 and addition of exogeneous calcium (as $CaCl_2$) to excised cotyledons. According to the experimental results, Chl accumulation was inhibited by Na_2EGTA and verapamil treatments. Nevertheless, application of ionophore A23187 also resulted in inhibition of Chl accumulation. Since Ca^{2+} is cytotoxic, uncontrolled increase in cytoplasmic calcium will result in expenditure of metabolic energy for pumping of Ca^{2+} out of cytoplasm and thus normal cellular processes are impaired. The addition of exogeneous Ca^{2+} (as $CaCl_2$) to excised cotyledons was an another way to resemble the natural increase in cytoplasmic [Ca^{2+}]. Different quantities of Ca^{2+} as adjusted by concentration or pretreatment period were applied to excised cotyledons, and their effects on Chl accumulation were determined. From the experimental results, low concentrations of Ca^{2+} slightly promoted Chl accumulation but as concentration increased Chl content decreased. However, the Ca^{2+} stimulation effect was shown to be more pronounced when duration of dark pretreatment decreased. Actually, the effect of Ca^{2+} on Chl accumulation in excised cotyledons is hardly demonstrated by experimental generation of increases in cytoplasmic Ca^{2+}. Therefore, Na_2EGTA treatment and verapamil treatment become the strongest evidences in supporting the involvement of Ca^{2+} in Chl accumulation. Besides, the TFP inhibition

effect further suggested the involvement of Ca^{2+} and calmodulin in Chl accumulation.

Indeed, calcium is now widely appreciated as second messenger in plant cells (5, 11, 12, 26, 39, 40, 52, 55, 56, 57 & 71) . The low intracellular concentration of Ca^{2+}, a wide range of stimuli-induced changes in cytoplasmic concnetration, the functional ubiquity of calmodulin and the Ca^{2+}-dependent protein kinase regulatory system; all of these inferred calcium to be involved in many physiological processes and signal transduction systems. In Chl accumulation as well, calcium is supposed to be the intracellular messenger of light signal and regulates Chl accumulation through a Ca^{2+} and calmodulin activated protein kinase regulatory system.

Table 4.1. Effect of benzyladenine (BA) on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with BA of different concentrations for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Mean values with SE were shown in columns, and means within columns followed by the same letter are not significantly different at P=0.05 according to Student-Newman-Keuls test. Experiment was done twice with 4 to 7 replicates in each treatments.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
$5x10^{-5}$ M BA	9.28±0.25*a*
$5x10^{-6}$ M BA	11.23±0.17*b*
$5x10^{-7}$ M BA	11.03±0.26*b*
$5x10^{-8}$ M BA	8.43±0.18*c*
water control	7.05±0.13*d*

Table 4.2. Effect of Na_2EGTA on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with Na_2EGTA of different concentrations for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR). Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legand to table 4.1.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
20 mM Na_2EGTA	1.38±0.05*a*
10 mM Na_2EGTA	1.30±0.04*a*
4 mM Na_2EGTA	4.12±0.11*b*
2 mM Na_2EGTA	5.49±0.17*c*
1 mM Na_2EGTA	6.03±0.16*d*
water control	7.62±0.16*e*

Table 4.3. Effect of $CaCl_2$ and Na_2EGTA on Chl accumulation in cucumber cotyledons excised from five-day-old plants into treatment solutions. The cotyledons were incubated in the dark for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legand to table 4.1.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
10 mM $CaCl_2$ + 10 mM Na_2EGTA	2.73±0.12*a*
4 mM $CaCl_2$ + 4 mM Na_2EGTA	7.52±0.14*b*
2 mM $CaCl_2$ + 2 mM Na_2EGTA	8.24±0.13*c*
1 mM $CaCl_2$ + 1 mM Na_2EGTA	8.43±0.17*c*
water control	6.85±0.11*d*

Table 4.4. Effect of $CaCl_2$+Na_2EGTA; NaCl on Chl accumulation in cucumber cotyledons excised from five-day-old plants into treatment solutions. The cotyledons were incubated in the dark for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legands to table 4.1.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
4 mM $CaCl_2$+Na_2EGTA	8.80±0.26*a*
8 mM NaCl	8.98±0.13*a*
0.5 mM $CaCl_2$+Na_2EGTA	8.90±0.13*a*
1 mM NaCl	9.10±0.17*a*
water control	7.97±0.13*b*

Table 4.5. Effects of $CaCl_2$ and Na_2EGTA on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were pretreated for 18 h in the dark and then exposed to light for 16 h (50 $uE.m^{-2}.s^{-1}$ PAR). Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legands to table 4.1.

Treatment	Chl content (ug Chl/cotyledon)
5 mM $CaCl_2$ + 4 mM Na_2EGTA	8.41±0.23*a*
4 mM $CaCl_2$ + 4 mM Na_2EGTA	10.28±0.31*b*
2 mM $CaCl_2$ + 4 mM Na_2EGTA	7.98±0.08*a*
1 mM $CaCl_2$ + 4 mM Na_2EGTA	6.49±0.08*c*
water control	8.25±0.24*a*

Table 4.6. Effect of NaCl on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with NaCl of different concentrations for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legand to table 4.1.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
10 mM NaCl	8.93±0.17*a*
4 mM NaCl	9.15±0.14*a*
2 mM NaCl	9.30±0.31*a*
1 mM NaCl	9.36±0.14*a*
0.5 mM NaCl	8.91±0.11*a*
water control	7.80±0.12*b*

Table 4.7a. Effect of $CaCl_2$ on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with $CaCl_2$ of different concentrations for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legand in table 4.1.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
10 mM $CaCl_2$	4.61±0.12*a*
5 mM $CaCl_2$	5.56±0.13*b*
1 mM $CaCl_2$	6.73±0.14*c*
0.1 mM $CaCl_2$	7.17±0.11*d*
water control	6.85±0.12*c*

Table 4.7b. Effect of $CaCl_2$ on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with $CaCl_2$ of different concentrations for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legand to table 4.1.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
100 uM $CaCl_2$	8.03±0.19*a*
50 uM $CaCl_2$	7.91±0.14*a*
10 uM $CaCl_2$	8.22±0.14*a*
5 uM $CaCl_2$	7.91±0.16*a*
1 uM $CaCl_2$	8.02±0.11*a*
water control	7.79±0.12*a*

Table 4.8. Effect of KCl on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with KCl of different concentrations for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legand to table 4.1.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
20 mM KCl	14.58±0.23*a*
10 mM KCl	15.48±0.18*b*
4 mM KCl	16.57±0.21*c*
2 mM KCl	14.96±0.21*ab*
1 mM KCl	14.76±0.27*ab*
water control	7.75±0.16*d*

Table 4.9. Ionic strength effects of KCl, NaCl, and $CaCl_2$ on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with the test solutions for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legand to table 4.1.

Ionic concentration	Ionic species	Chl content (ug Chl/cotyledon)
$1x10^{-5}$ M	10 uM KCl	7.82±0.18*a*
	10 uM NaCl	8.41±0.14*b*
	3.3 uM $CaCl_2$	8.15±0.31*ab*
	water control	7.04±0.13*c*
$1x10^{-4}$ M	100 uM KCl	10.04±0.23*a*
	100 uM NaCl	8.72±0.40*b*
	33.3 uM $CaCl_2$	7.69±0.13*c*
	water control	7.04±0.13*d*
$1x10^{-3}$ M	1 mM KCl	13.63±0.24*a*
	1 mM NaCl	9.50±0.21*b*
	0.33 mM $CaCl_2$	7.69±0.11*c*
	water control	7.04±0.13*d*
$1x10^{-2}$ M	10 mM KCl	13.90±0.26*a*
	10 mM NaCl	8.80±0.24*b*
	3.3 mM $CaCl_2$	6.86+0.12*c*
	water control	7.04±0.13*c*

Table 4.10a. Effects of application of KCl, NaCl, and $CaCl_2$ for different time intervals during dark pretreatment and illumination on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with the test solutions for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legand to table 4.1.

Pretreatment	Illumination	Chl content (ug Chl/cotyledon)
KCl(9h)+H_2O(9h)	H_2O(16h)	14.99±0.32*a*
KCl(15h)+H_2O(3h)	H_2O(16h)	15.84±0.32*a*
KCl(18h)	H_2O(16h)	17.83±0.50*b*
H_2O(18h)	KCl(16h)	17.13±0.44*b*
H_2O(18h)	H_2O(16h)	9.13±0.27*c*
NaCl(9h)+H_2O(9h)	H_2O(16h)	10.75±0.19*a*
NaCl(15h)+H_2O(3h)	H_2O(16h)	10.70±0.26*a*
NaCl(18h)	H_2O(16h)	10.71±0.55*a*
H_2O(18h)	NaCl(16h)	11.16±0.19*a*
H_2O(18h)	H_2O(16h)	9.13±0.27*b*
$CaCl_2$(9h)+H_2O(9h)	H_2O(16h)	10.72±0.25*a*
$CaCl_2$(15h)+H_2O(3h)	H_2O(16h)	10.08±0.20*b*
$CaCl_2$(18h)	H_2O(16h)	9.66±0.05*bc*
H_2O(18h)	$CaCl_2$(16h)	9.49±0.07*bc*
H_2O(18h)	H_2O(16h)	9.13±0.27*c*

Remarks: KCl : 4 mM
NaCl : 1 mM
$CaCl_2$: 10 uM

Table 4.10b. Effects of application of KCl, NaCl, and $CaCl_2$ for different time intervals during dark pretreatment and illumination on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with the test solutions for 18 h and then exposed to light (50 uEm^{-2} s^{-1} PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legand to table 4.1.

Pretreatment	Illumination	Chl content (ug Chl/cotyledon)
KCl(9h)+H_2O(9h)	H_2O(16h)	14.09±0.32*a*
NaCl(9h)+H_2O(9h)	H_2O(16h)	10.75±0.19*b*
$CaCl_2$(9h)+H_2O(9h)	H_2O(16h)	10.72±0.25*b*
H_2O(18)	H_2O(16h)	9.13±0.27*c*
KCl(15h)+H_2O(3h)	H_2O(16h)	15.84±0.32*a*
NaCl(15h)+H_2O(3h)	H_2O(16h)	10.70±0.26*b*
$CaCl_2$(15h)+H_2O(3h)	H_2O(16h)	10.08±0.20*b*
H_2O(18h)	H_2O(16h)	9.13±0.27*c*
KCl(18h)	H_2O(16h)	17.83±0.50*a*
NaCl(18h)	H_2O(16h)	10.71±0.55*b*
$CaCl_2$(18h)	H_2O(16h)	9.66±0.05*bc*
H_2O(18h)	H_2O(18h)	9.13±0.27*c*
H_2O(18h)	KCl(16h)	17.13±0.44*a*
H_2O(18h)	NaCl(16h)	11.16±0.19*b*
H_2O(18h)	$CaCl_2$(16h)	9.49±0.07*c*
H_2O(18h)	H_2O(16h)	9.13±0.27*c*

Remark: KCl : 4 mM
NaCl : 1 mM
$CaCl_2$: 10 uM

Table 4.11. Effect of verapamil on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with verapamil of different concentrations for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legand to table 4.1.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
5000 uM verapamil	0.83±0.10*a*
1000 uM verapamil	0.86±0.06*a*
100 uM verapamil	4.72±0.18*b*
10 uM verapamil	7.03±0.16*c*
1 uM verapamil	7.98±0.10*d*
water control	7.92±0.30*d*

Table 4.12a. Effect of calcium ionophore A23187 on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with ionophore A23187 of different concentrations for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legand to table 4.1.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
20 uM A23187	1.24±0.05*a*
10 uM A23187	1.10±0.05*a*
5 uM A23187	2.51±0.10*b*
1 uM A23187	6.41±0.14*c*
water control	8.05±0.17*d*

Table 4.12b. Effect of calcium ionophore A23187 on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with ionphore A23187 of different concentrations for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legand to table 4.1.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
500 nM A23187	5.72±0.13*a*
200 nM A23187	5.65±0.19*a*
100 nM A23187	6.97±0.18*b*
50 nM A23187	7.12±0.24*b*
water control	8.00±0.25*c*

Table 4.13. Effect of trifluoperazine (TFP) on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with TFP of different concentrations for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in the legand to table 4.1.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
200 uM TFP	0.70±0.04*a*
150 uM TFP	2.25±0.10*b*
100 uM TFP	4.30±0.11*c*
50 uM TFP	6.12±0.12*d*
10 uM TFP	7.61±0.13*e*
water control	7.52±0.16*e*

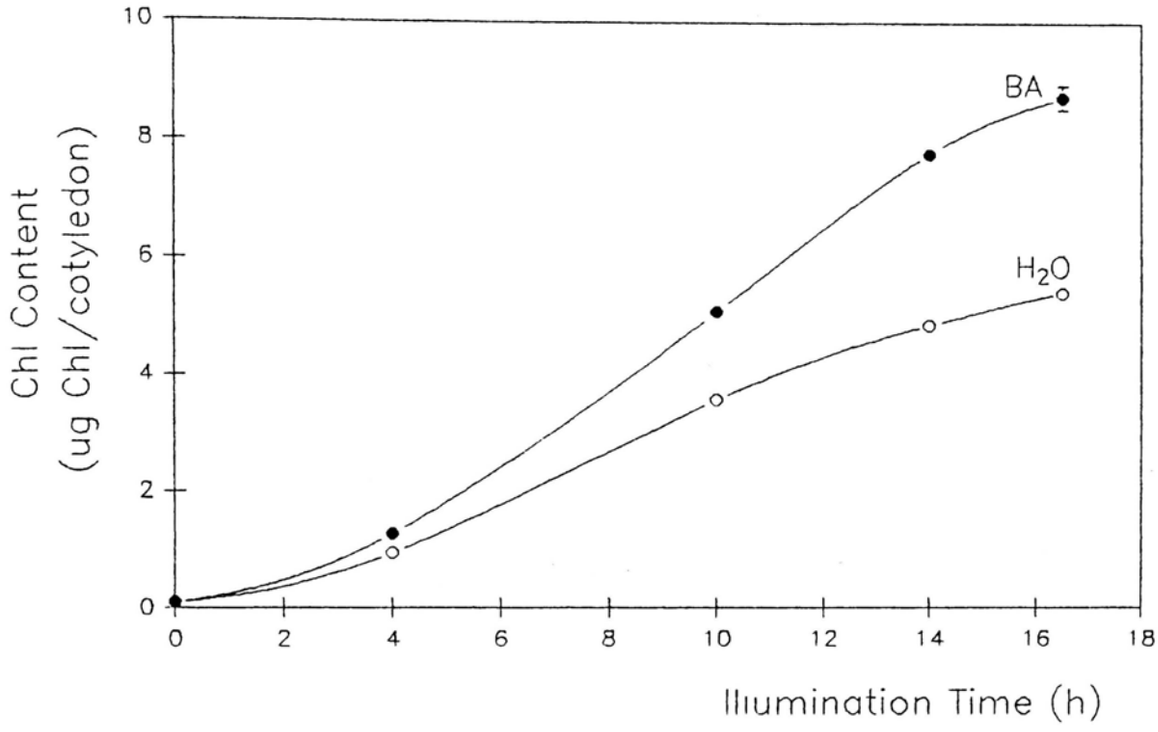

Figure 4.1. Time course of Chl accumulation under continuous illumination in BA-treated cotyledons and water control. Cotyledons were dark pretreated with BA for 18 h and then exposed to light (50 uE $m^{-2}s^{-1}$ PAR) for various periods. Chl content of 10 ml of 80% acetone extract of ten cotyledons was expressed as ug Chl/cotyledon. Each point represented the mean of 8 replicate samples.

Chapter 5

The Calcium Effect on Benzyladenine-induced Stimulation of Chlorophyll Accumulation in Excised Cucumber Cotyledons

Introduction

Calcium is now widely recognised as an important regulatory molecule in higher plants (5, 26 & 39). Certain phytochrome-mediated processes and cytokinin-regulated responses in plant cells are reported to involve changes in cytoplasmic free calcium (12, 60 & 62). Stimulation of Chl accumulation by BA in excised cucumber cotyledons is well documented (2, 13, 14, 15 & 16). Through the enhancement of ALA synthesis and promotion of expression of cab gene encoding the light harvesting Chl *a/b* binding protein by BA, stimulation of Chl accumulation is resulted (15, 53). However, nothing is known regarding the exact molecular mechanism of the BA action. As treatment of BA-treated plant materials with calmodulin antagonist inhibited the BA-regulated responses (19, 20), it is proposed that calcium, and probably calcium-calmodulin complex are involved in cytokinin-regulated responses. Consequently, the present investigation was undertaken to demonstrate the involvement of calcium and calmodulin in BA-stimulated Chl accumulation in excised cucumber cotyledons.

Materials and Methods

Materials

Cucumber seeds (*Cucumis sativus* L. cv. Sure Green) were obtained from Known-You Seed Co., Ltd., Taiwan

The following chemicals were purchased from Sigma: benzyladenine, EGTA, TFP and verapamil. Acetone was obtained from Mallinckrodt.

Methods

Plant growth and treatment

Cucumber seeds were soaked in distilled water for 15 min before planted in vermiculite which had been watered once with 250 ml distilled water. Seeds were germinated in the dark at 28°C for 5 days. At the day of harvesting, cotyledons were excised under dim green light and were placed into conical flasks containing 25 ml of test solutions. Dark pretreatment was carried at 28°C for 18 h with the conical flask kept in orbital shaking at 100 rpm. The test solutions included 5 uM BA, 4 mM Na_2EGTA (a calcium chelator), 100 uM verapamil (calcium channel blocker) and 100 uM trifluoperazine (TFP, a calmodulin antagonist).

Pretreatment experiment

In pretreatment experiment, Na_2EGTA, verapamil and TFP

were applied to BA-treated cotyledons during dark pretreatment respectively. After 18 h dark preincubation, cotyledons were transferred to petri dishes containing 25 ml of test solutions and were illuminated under the light intensity at 50 $uEm^{-2}s^{-1}$ PAR for 16 h. Chl of ten cotyledons was extracted with 10 ml of 80% acetone and the homogenate was centrifuged at 3000 *g* for 15 min. Chl content in the supernatant was determined by measuring absorbance at 663 nm and was expressed as ug Chl/cotyledon.

Sequence experiment

In sequence experiment, the test solutions were applied in different sequences to the excised cotyledons during dark pretreatment. Afterwards, cotyledons were transferred to petri dishes containing 25 ml of distilled water and were illuminated for 16 h. Chl content of ten cotyledons was determined as previously described.

Result

Pretreatment experiment

The results of pretreatment experiment were shown in tables 5.1, 5.2 & 5.3. BA-stimulated Chl accumulation in excised cotyledons of cucumber cultivar Sure Green was confirmed. Treatment of excised cotyledons with 5 uM BA solution resulted in 65% to 77% increase in Chl

accumulation. Application of 4 mM Na_2EGTA, 100 uM verapamil and 100 uM TFP to excised cotyledons inhibited Chl synthesis, resulting in 42% to 47% inhbition of Chl accumulation. Treatment of BA-treated cotyledons with Na_2EGTA, verapamil and TFP abolished the BA stimulation effect. Chl content of (BA+Na_2EGTA)-treated cotyledons was 77% of water control and that of (BA+verapamil)-treated cotyledons was 84%. For (BA+TFP)-treated cotyledons, Chl content was 48% of water control. It seemed that the BA stimulation effect was calcium and calmodulin-dependent.

Sequence experiment

In sequence experiment, test solutions were applied in different sequences to the excised cotyledons during pretreatment and resulted in various degree of stimulation and inhibition of Chl accumulation (Tables 5.4, 5.5 & 5.6). BA stimulation of Chl accumulation was also demonstrated in sequence experiment. The stimulation effect was more pronounced in cotyledons pretreated with BA and then H_2O than those pretreated with H_2O and then BA solution. Chl content of BA-H_2O-treated cotyledons was about 160% of water control and that of H_2O-BA-treated cotyledons was 139%. Although the exposure time of the cotyledons to BA solution was the same in both sequences (9h in the dark), the retention time of the hormone inside the plant cells

was actually different. Prolonged retention of BA inside the cells resulted in more pronounced stimulation effect (Tables 5.4, 5.5 & 5.6). For the Na_2EGTA inhibition effect, Chl content of H_2O-Na_2EGTA-treated cotyledons was 77% of water control and in Na_2EGTA-H_2O-treated cotyledons, Chl content was 55% of water control. Prolonged retention of Na_2EGTA inside the cells resulted in greater inhibition of Chl accumulation. Application of Na_2EGTA to BA-pretreated cotyledons inhibited the BA stimulation efffect, resulting in 8% increase in Chl accumulation. To determine whether the Na_2EGTA inhibition effect can be reserved by BA, BA was applied to Na_2EGTA-pretreated cotyledons. Chl content of Na_2EGTA-BA-treated cotyledons was 55% of water control. Inhibition in Na_2EGTA-BA-treated cotyledons was much greater than that in BA-Na_2EGTA-treated cotyledons.

Inhibition by verapamil of Chl accumulation was also demonstrated in sequence experiment (Table 5.5). H_2O-verapamil-treated cotyledons and verapamil-H_2O-treated cotyledons had the same Chl content of 80% of water control. It showed that verapamil inhibition effect was dependent on the exposure time of cotyledons to verapamil rather than the retention time of the channel blocker inside the cells. Application of verapamil to BA-pretreated cotyledons inhibited the BA stimulation effect, resulting in only 18% increase in Chl accumulation. As mentioned

before, the BA stimulation effect in H_2O-BA-treated cotyledons was less than that in BA-H_2O-treated cotyledons. The application of BA to verapamil-pretreated cotyledons also resulted in 18% increase in Chl accumulation. The verapamil inhibition effect seemed to be reversed by the BA treatment.

TFP inhibited Chl accumulation in sequence experiment. Chl content of H_2O-TFP-treated cotyledons and TFP-H_2O-treated cotyledons was 68% and 66% of water control respectively. Prolonged retention of TFP inside the cells did not resulted in greater inhibition of Chl formation. The Chl content of BA-TFP-treated cotyledons was 77% of water control, that is the same as the Chl content of TFP-BA-treated cotyledons. The TFP inhibition effect seemed to be partially reversed by the BA treatment.

Discussion

Calcium is reported to involve in certain phytochrome-mediated processes and cytokinin-regulated responses in plant cells (12, 60 & 62) . The effects of calcium on chlorophyll accumulation in greening cotyledons and dark-induced senescent plant materials are studied extensively in the last ten years (32, 33, 64, 65 & 66). BA-induced stimulation of Chl accumulation in excised cotyledons of cucumber cultivar Sure Green was confirmed in the present

study. Chelation of cytoplasmic free calcium by Na_2EGTA and blockage of natural increase in cytoplasmic calcium by verapamil inhibited Chl synthesis. Treatemt with calmodulin antagonist TFP to excised cotyledons also resulted in inhibition of Chl accumulation. Therefore, it seems that calcium and calmodulin is probably involved in Chl accumulation in excised cucumber cotyledons. For the calcium effect on BA-induced stimulation of Chl accumualtion, Na_2EGTA, verapamil and TFP were applied to BA-treated cotyledons in pretreatment experiments. As Chl accumulation was inhibited by Na_2EGTA, verapamil and TFP treatments, determination the effects of the test solutions on the BA stimulation effect based on % of water control was inconclusive. Consequently, BA-dependence was introduced to determine the effects of the test solution on the BA-regulated response. BA-dependence was adopted from Elliott (19) and modified here, defining as 'a percentage used to measure the degree or level of the effects of the test solutions on the BA stimulation effect'. Low BA-dependence indicated inhibition effect of the test solution. Table 5.7 summarized the BA-dependences of various treatments. For pretreatment experiment, BA-dependences of (BA+Na_2EGTA)-treated cotyledons and (BA+verapamil)-treated cotyledons were 29% and 40% respectively. The BA stimulation effect was abolished by

Na_2EGTA and verapamil treatments. It is supposed that calcium and changes in cytoplasmic calcium may be involved in the BA effect. In addition, BA-dependence of (BA+TFP)-treated cotyledons was 0%. The TFP inhibition of the BA stimulation effect was the greatest among the other two treatments. The calmodulin-dependence of the BA action remains to be studied.

In sequence experiments, application of test solutions in different sequences to excised cotyledons resulted in various degree of stimulation and inhibition of Chl accumulation. For the Na_2EGTA effect, BA-dependence of BA-Na_2EGTA-treated cotyledons was 50%, which was higher than BA-dependence of (BA+Na_2EGTA)-treated cotyledons. However, BA-dependence of Na_2EGTA-BA-treated cotyledons was 0%. Removal of cytoplasmic calcium before BA administration totally inhibited the BA stimulation effect. The different in BA-dependence among various BA and Na_2EGTA treatments may be resulted from the retention time of the test solutions inside the cells. Prolonged retention of Na_2EGTA inside the cells resulted in higher degree of inhibition of Chl accumulation and the BA stimulation effect. Also, the BA stimulation effect was depended on retention time (Tables 5.3, 5.4 & 5.5).

Verapamil inhibited calcium-dependent processes through the blockage of calcium channel and preventing

increase in cytoplasmic calcium. As mentioned before, the verapamil inhibition effect was dependent on exposure time to verapamil rather than the retention time inside the cells. BA-dependence of BA-verapamil-treated cotyledons was 67%, whereas BA-dependence of verapamil-BA-treated cotyledons was 90%. The application of BA to verapamil-pretreated cotyledons reversed the verapamil inhibition effect. Consequently, it seems that there existed the antagonist between BA and verapamil: BA may induce an increase in cytoplasmic calcium while verapamil inhibits it. For the TFP effect, BA-dependences of BA-TFP-treated cotyledons and TFP-BA-treated cotyledons were 17% and 25% respectively. The TFP inhibition effect seemed to be partially reversed by the BA treatment. However, the role of calmodulin in the BA action is not known.

From both of pretreatment and sequence experiments, chelation and blockage of increase in cytoplasmic calcium abolished the BA stimulation effect. In addition, the BA effect was inhibited by calmodulin antagonist. Consequently, it is proposed that calcium may be involved in the BA-induced stimulation of Chl accumulation and probably through a calmodulin-dependent mechanism. Actually, there is increasing interest in defining the possible role of calcium as intracellular messenger of light and hormonal signals in higher plants (5, 40 & 57).

In the present study, calcium and calmodulin are demonstrated to be involved in Chl synthesis and the BA-induced stimulation effect. However, the precise calcium-modulated biochemical reactions involved in the Chl biosynthetic pathway and the transduction chains of light and hormonal signals are not known yet. Further investigation on the molecular mechanisms of light and BA actions is a requisite for defining the role of calcium in these signal transduction processes.

Table 5.1. Effect of BA, Na_2EGTA on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with the test solutions for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Mean values with the SE were shown in columns, and means within columns followed by the same letter are not significantly different at P=0.05 according to Student-Newman-Keuls test. All treatments had 4 to 5 replicates.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
$5x10^{-6}$ M BA + 4 mM Na_2EGTA	5.96±0.25*a*
$5x10^{-6}$ M BA	12.77±0.24*b*
4 mM Na_2EGTA	4.50±0.19*c*
water control	7.74±0.19*d*

Table 5.2. Effect of BA, verapamil on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with the test solutions for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in table 5.1.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
$5x10^{-6}$ M BA + 100 uM verapamil	7.05±0.23*a*
$5x10^{-6}$ M BA	14.79±0.29*b*
100 uM verapamil	4.49±0.17*c*
water control	8.36±0.19*d*

Table 5.3. Effect of BA, trifluoperazine on Chl accumulation in cucumber cotyledons excised from five-day-old plants. Cotyledons were dark pretreated with the test solutions for 18 h and then exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 16 h. Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in table 5.1.

Treatment	Chl content (ug Chl/cotyledon) after 16 h illumination
$5x10^{-6}$ M BA + 100 uM TFP	4.01±0.17*a*
$5x10^{-6}$ M BA	14.79±0.29*b*
100 uM TFP	4.44±0.13*a*
water control	8.36±0.19*c*

Table 5.4. Effect of treatment of excised cucumber cotyledons with H_2O, BA, Na_2EGTA for different time intervals in the dark on Chl accumulation. Cotyledons were excised from five-day-old plants into the test solutions and were incubated in the dark for 18 h. During illumination, cotyledons were transferred to H_2O and were exposed to light (50 $uEm^{-2}s^{-1}$ PAR). Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Mean values with the SE was shown in columns, and means within columns followed by the same letter are not significantly different at P=0.05 according to Student-Newman-Keuls test. Experiment was repeated twice with 8 replicates in each treatments.

Treatment in darkness 9 h	9 h	Chl content (ug Chl/cotyledon) after 16 h illumination
H_2O	Na_2EGTA	5.71±0.13*a*
BA	Na_2EGTA	7.98±0.18*b*
BA	H_2O	11.99±0.26*c*
Na_2EGTA	H_2O	4.07±0.12*d*
Na_2EGTA	BA	3.93±0.17*d*
H_2O	BA	10.30±0.27*c*
H_2O	H_2O	7.42±0.07*b*

Remark : BA: $5x10^{-6}$ M
Na_2EGTA: 4 mM

Table 5.5. Effect of treatment of excised cucumber cotyledons with H_2O, BA, verapamil for different time intervals in the dark on Chl accumulation. Cotyledons were excised from five-day-old plants into the test solutions and were incubated in the dark for 18 h. During illumination, cotyledons were transferred to H_2O and were exposed to light (50 $uEm^{-2}s^{-1}$ PAR). Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in table 5.4.

Treatment in darkness 9 h	9 h	Chl content (ug Chl/cotyledon) after 16 h illumination
H_2O	verapamil	7.28±0.13*a*
BA	verapamil	10.85±0.26*b*
BA	H_2O	14.32±0.37*c*
verapamil	H_2O	7.56±0.16*a*
verapamil	BA	10.80±0.27*b*
H_2O	BA	12.79±0.35*d*
H_2O	H_2O	9.18±0.13*e*

Remark : BA: $5x10^{-6}$ M
verapamil: 100 uM

Table 5.6. Effect of treatment of excised cucumber cotyledons with H_2O, BA, TFP for different time intervals in the dark on Chl accumulation. Cotyledons were excised from five-day-old plants into the test solutions and were incubated in the dark for 18 h. During illumination, cotyledons were transferred to H_2O and were exposed to light (50 $uEm^{-2}s^{-1}$ PAR). Chl content of 10 ml acetone extract of 10 cotyledons was expressed as ug Chl/cotyledon. Other notes were as in table 5.4.

Treatment in darkness 9 h	9 h	Chl content (ug Chl/cotyledon) after 16 h illumination
H_2O	TFP	6.09±0.11*a*
BA	TFP	6.91±0.19*b*
BA	H_2O	13.90±0.32*c*
TFP	H_2O	5.92±0.14*a*
TFP	BA	6.80±0.16*b*
H_2O	BA	12.59±0.29*d*
H_2O	H_2O	9.02±0.11*e*

Remark : BA: $5x10^{-6}$ M
TFP: 100 uM

Table 5.7. Table summaried the BA-dependences of various treatments in both pretreatment experiments and sequence experiments.

Experiment	Treatment	BA-dependence
Pretreatment experiments		
	BA	100 %
	BA+Na_2EGTA	29 %
	BA	100 %
	BA+verapamil	40 %
	BA	100 %
	BA+TFP	0 %
Sequence Experiments:		
	BA (9) + H_2O (9)	100 %
	BA (9) + Na_2EGTA (9)	50 %
	H_2O (9) + BA (9)	100 %
	Na_2EGTA (9) + BA (9)	0 %
	BA (9) + H_2O (9)	100 %
	BA (9) + verapamil (9)	67 %
	H_2O (9) + BA (9)	100 %
	verapamil (9) + BA (9)	90 %
	BA (9) + H_2O (9)	100 %
	BA (9) + TFP (9)	17 %
	H_2O (9) + BA (9)	100 %
	TFP (9) + BA (9)	25 %

Remarks: BA : $5x10^{-6}$ M
Na_2EGTA : 4 mM
verapamil : 100 uM
TFP : 100 uM

Chapter 6

Regulation of 5-Aminolevulinic Acid and Chlorophyll Accumulation in Levulinic Acid-treated Cotyledons

Introduction

5-Aminolevulinic acid (ALA) is known to play a pivotal role on the tetrapyrrole biosynthetic pathway leading to Chl and heme synthesis (4, 10 & 35). In the initial stage of greening, ALA formation is the limiting step and ALA synthesizing enzymes are made *de novo* (10, 37). Preillumination of etiolated plant materials, or dark pretreatment with cytokinin triggered the formation of ALA synthesizing system and thereby eliminated the lag phase of Chl formation after the onset of illumination (10, 15, 37 & 42). It is known that light regulates ALA synthesis by exerting coordinated transcriptional control over the ALA synthesizing enzymes (34). In addition, cytokinin is known to stimulate Chl accumulation through the enhancement of ALA synthesis (10, 15 & 42). It is proposed that cytokinin may rapidly and specifically initiate synthesis of ALA synthesizing enzymes in the dark and mimicking the light effect on ALA formation (42).

Levulinic acid, a competitive inhibitor of ALA dehydrase that caused ALA accumulation and inhibited Chl synthesis, is extensively used in the studies of Chl

biosynthetic pathway and ALA accumulation (10, 15, 37 & 42). In this study, ALA accumulation as another parameter of greening, is selected to investigate the regulatory effects and site of action of BA, Na^+, Ca^{2+} and K^+ on greening process.

Materials and Methods

Materials

Cucumber seeds (*Cucumis sativus* L. cv. Sure Green) was obtained from Known You Seed Co.,Ltd., Taiwan.

The following were purchased from Sigma: BA, EGTA, $CaCl_2$, KCl, verapamil, TFP and p-dimethylaminobenzaldehyde (DMAB). NaCl was obtained from Anala R. Acetone was from Mallinckrodt; acetylacetone was from Peking Chemical Work. Trichloroacetic acid was obtained from Ajax chemicals. Glacial acetic acid was from Merck.

Methods

Plant growth and treatment

Cucumber seeds were soaked in distilled water for 15 min before planted in vermiculite which had been watered once with 250 ml distilled water. The seeds were germinated in the dark at 28°C for 5 days. At the day of harvesting, cotyledons were excised without their hypocotyl hooks and

eighty cotyledons were placed into a conical flask containing 25 ml of test solution. All manipulation described above was performed under dim green safelight. Dark preincubination was carried out at 28°C for 15 h with the conical flasks kept in orbital shaking at 100 rpm. After 15 h of dark pretreatment, cotyledons were transferred to conical flasks containing 25 ml of levulinic acid (LA) solution (pH adjusted to 6.8 with NaOH) and were incubated in the dark for 3 h. Afterwards, cotyledons were transferred to petri dishes containing 25 ml of LA solution and were illuminated at 50 $uEm^{-2}s^{-1}$ PAR.

Chl determination and ALA assay

Chl content of ten LA-treated cotyledons was extracted with 10 ml of 80% acetone and the homogenate was centrifuged at 3000 *g* for 15 min. The Chl content in the supernatant was determined by measuring absorbance at 663 nm and was expressed as ug Chl/cotyledon or nmol ALA equivalent/cotyledon.

For the determination of ALA, each twenty cotyledon samples was homogenized with 10 ml of 4% (w/v) trichloroacetic acid and the homogenate was centrifuged at 3000 *g* for 15 min. And then, 0.5 ml of supernatant (ALA extract) was diluted with 1.5 ml of 4% (w/v) trichloroacetic acid, and was added to 0.94 ml of 1 M

sodium acetate and 0.06 ml of acetylacetone. The mixture was heated at 100°C for 15 min. Afterwards, equal aliquots of the sample and modified Ehrlich's reagent (1 gm of DMAB in 10 ml of 70% perchloric acid and 40 ml of glacial acetic acid) were mixed (45). Fifteen min later, the ALA content in the reaction mixture was determined by measuring absorbance at 554 nm against a control which had been treated identically but where acetylacetone was replaced by distilled water. The ALA content which expressed as nmol ALA/cotyledon was calculated from a standard curve with samples containing known amount of ALA.

Measurement of ALA and Chl accumulation in LA-treated cotyledons

As described in plant growth and treatment, cucumber seeds were germinated in the dark for 5 days. At the day of harvesting, cotyledons were excised and were dark pretreated with distilled water for 15 h and then treated with distilled water or LA solutions of concentrations at 20, 50, 100 and 200 mM (pHs adjusted to 6.8 with NaOH) for 3 h. Afterwards, the cotyledons were illuminated at 50 $uEm^{-2}s^{-1}$ PAR for 10 h. The ALA and Chl content in LA-treated cotyledons was determined as previously described.

ALA accumulation curve

Cucumber seeds were germinated in the dark for 5 days.

At the day of harvesting, cotyledons were excised and were dark pretreated with distilled water for 15 h and then treated with 50 mM LA solution (pH adjusted to 6.8 with NaOH) for 3 h. Afterwards, the LA-treated cotyledons were illuminated for 0, 4, 8, 10, 12, 14, 16 and 18 h. The ALA and Chl content in LA-treated cotyledons was determined as previously described.

Measurement of Chl accumulation in 50 mM NaCl-treated cotyledons

In ALA accumulation experiments, LA was administrated as sodium levulinate since pH of the solution was adjusted with NaOH. As demonstrated before, Na^+ has regulatory effect on Chl accumulation. Consequently, the effect of Na^+ as administrated 3 h before the onset of illumination and during illumination on Chl accumulation was investigated.

Cucumber seeds were dark germinated for 5 days. Afterwards, cotyledons were excised and were dark pretreated with distilled water for 15 h and then treated with 50 mM NaCl (which was equivalent to the Na^+ content in 50 mM LA solution) for 3 h. And then, the cotyledons were transferred to petri dishes containing 25 ml of 50 mM NaCl and were illuminated for 0, 4 and 10 h. Chl content of ten cotyledons was extracted with 10 ml of 80% acetone and the homogenate was centrifuged at 3000 *g* for 15 min. The Chl content in the supernatant was determined by measuring

absorbance at 663 nm and was expressed as ug Chl/cotyledon.

In addition, the 50 mM NaCl effect on Chl accumulation in BA-, cations-, Na_2EGTA-, verapamil- and TFP-pretreated cotyledons was also examined.

Measurement of ALA and Chl accumulation in BA-, cations-, Na_2EGTA-, verapamil- and TFP-pretreated cotyledons

As described in plant growth and treatment, cucumber seeds were germinated in the dark for 5 days. At the day of harvesting, cotyledons were excised and were dark pretreated with the test solutions for 15 h and then treated with 50 mM LA solution (pH adjusted to 6.8 with NaOH) for 3 h. Afterwards, the LA-treated cotyledons were illuminated for 0, 4 and 10 h. The ALA and Chl content in LA-treated cotyledons was determined as previously described. The test solutions in this study included 5 uM BA, 4 mM KCl, 1 mM NaCl, 10 uM $CaCl_2$, 4 mM Na_2EGTA, 100 uM verapamil and 100 uM TFP.

Results

Concentrations effect of LA on ALA and Chl accumulation

Table 6.1 presented the concentrations effect of LA on ALA and Chl accumulation in excised cucumber cotyledons. LA concentrations at 0 (water control), 20, 50, 100 and 200 mM were tested. Only a trace amount of ALA was accumulated in the absence of LA (water control). LA solution caused ALA

accumulation and inhibited Chl synthesis. As compared with water control, 20 mM LA solution increased ALA content by 35-fold and inhibited Chl accumulation by 29%. 50 mM LA solution increased ALA content by 90-fold and decreased Chl content by 78%. However, for 100 mM LA-treated cotyledons ALA and Chl contents were 33-fold and 15% of water control respectively. 200 mM LA solution caused 11-fold increase in ALA accumulation and decreased Chl content by 93%. It was observed that as concentrations of LA solution increased ALA content increased and Chl content decreased, but a marked reduction in ALA content was found at high LA concentration. Dei have mentioned that prolonged treatment of excised cotyledons with LA solution was inhibitory (15). The inhibition of ALA accumulation by high LA concentrations may be due to the side effects of LA administrated or the accumulated ALA itself. Another unique feature observed in 20 mM and 50 mM LA-treated cotyledons was the amount of ALA accumulated more than the decrease in Chl content. It seems that not all ALA accumulated was destined for Chl formation. Among the LA concentrations tested, 50 mM LA solution having a greater inhibition of ALA conversion to Chl was chosen for the ALA accumulation experiments.

ALA accumulation curve

The ALA accumulation curve was shown in figure 6.1. As with greening curve, ALA accumulation possessed a lag phase during the initial 2 h of illumination and a steady-state rate of ALA formation. Moreover, a stationary phase of ALA accumulation was observed after 12 h of illumination. Chl content in LA-treated cotyledons also increased with illumination period. At 4 h of illumination, Chl content which expressed as nmol ALA equivalent /cotyledon was 3 nmol and increased to 11 nmol as illumination proceeded. However, when Chl content was expressed on the basis of % of ALA+Chl content at different illumination periods, it decreased from 23% at 4 h to 17% at 12 h of illumination. Dei reported that the decrease in Chl accumulation as a result of prolonged exposure to LA solution may be due to the inhibition of thylakoid Chl-proteins synthesis and assembly by LA and thereby inhibited Chl accumulation (15). Consequently, illumination periods from 0 to 10 h were selected for the time course experiments.

Effect of 50 mM NaCl on Chl accumulation

As demonstrated before, Na^+ has regulatory effect on Chl accumulation in excised cucumber cotyledons. In ALA accumulation experiments, LA solution was administrated as sodium levulinate and therefore, NaCl treatment was also used as control.

The 50 mM NaCl effect on Chl accumulation in water-pretreated cotyledons was shown in table 6.2. 50 mM NaCl as administrated 3 h before the onset of illumination and during illumination inhibited Chl accumulation during the light periods studied. At 4 h of illumination, 50 mM NaCl decreased Chl content by 22%. The Chl content was decreased by 27% at 10 h of illumination. The 50 mM NaCl inhibition effect was also demonstrated in excised cotyledons pretreated with the test solutions.

The 50 mM NaCl effect on Chl accumulation in BA-pretreated cotyledons was shown in table 6.3. 50 mM NaCl inhibited Chl accumulation in both BA-pretreated and water-pretreated cotyledons. At 4 h of illumination, BA stimulated Chl accumulation by 35 % in BA-H_2O-treated cotyledons and 31% in BA-NaCl-treated cotyledons (Table 6.10). The BA stimulation effect was decreased by the NaCl treatment to 29% at 10 h of illumination in contrast to the 44% stimulation in the absence of 50 mM NaCl. The inhibition of the BA effect by the NaCl treatment was more pronounced at prolonged illumination period. For the cations, Na^+, Ca^{2+} and K^+ stimulated Chl accumulation but the stimulation effects were decreased by the NaCl treatment (Tables 6.4, 6.5, 6.6 and 6.10). Na^+ stimulated Chl accumulation by 8% at 4 h and 23% at 10 h of illumination. In the presence of 50 mM NaCl, the

stimulation effect of Na^+ was decreased to 5% and 11% at 4 and 10 h respectively. Similarly, Ca^{2+} stimulated Chl accumulation by 10% and 23% at 4 and 10 h of illumination but the stimulation effect was decreased to 6% and 9% by the NaCl treatment. K^+ increased Chl content by 33% in KCl-H_2O-treated cotyledons and 31% in KCl-NaCl-treated cotyledons at 4 h of illumination. When illumination period prolonged to 10 h, K^+ stimulated Chl accumulation by 77% but the stimulation effect was decreased to 34% by the NaCl treatment. The 50 mM NaCl inhibition effect was more pronounced at prolonged illumination period.

The effect of 50 mM NaCl on Chl accumulation in Na_2EGTA-pretreated cotyledons was shown in table 6.7. Na_2EGTA inhibited Chl accumulation by about 50% during the light period studied. The presence of 50 mM NaCl only slightly increased the inhibition effect on Chl accumulation although the NaCl treatment decreased Chl content in both Na_2EGTA-pretreated and water-pretreated cotyledons (Tables 6.7 and 6.11). Verapamil inhibited Chl accumulation by 30% at 4 h and 40% at 10 h of illumination (Table 6.8). Treatment of the verapamil-pretreated cotyledons with 50 mM NaCl did not further increase the inhibition effect (Table 6.11). The 50 mM NaCl effect on Chl accumulation in TFP-pretreated was shown in table 6.9. TFP decreased Chl content by 50% and the inhibition effect

was not further increased by the NaCl treatment (Table 6.11). It was found that the inhibition effects of Na_2EGTA, verapamil and TFP on Chl accumulation was affected in a less extent than the stimulation effects of BA and the cations by the NaCl treatment.

Table 6.12 showed the comparsion between the % of water control and the % of NaCl control in various treatments. As in the ALA accumulation experiments, the presence of Na^+ in LA solution cannot be excluded. Consequently, the influence of Na^+ on the effects of BA and the cations, as well as water control on ALA and Chl accumulation in LA-treated cotyledons must be taken into consideration and the experimental results were compared with the % of NaCl control.

Effect of BA on ALA and Chl accumulation in LA-treated cotyledons

Table 6.13 showed the effect of BA on ALA and Chl accumulation in LA-treated cotyledons. At the onset of illumination (0 h), the ALA and Chl contents were the same in both BA-pretreated and water-pretreated cotyledons. As illumination proceeded, BA stimulated ALA and Chl accumulation. At 4 h of illumination, BA increased ALA content by 40% and Chl content by 29%. By comparing the ALA+Chl content in BA-pretreated and water-pretreated cotyledons, BA stimulated ALA+Chl accumulation by 38%. As

expected from the % of NaCl control, the BA stimulation effect would be 40% at 10 h of illumination (Table 6.12). However, it was found that BA stimulated ALA+Chl accumulation only by 29% during a 10-h light period. The ceased increase in BA stimulation effect may imply that there existed a feedback inhibition mechanism of ALA synthesizing activity by the accumulated ALA itself. When ALA accumulated to a certain quantity, the feedback inhibition mechanism began to operate and ALA accumulation slowed down.

Effects of Na^+, Ca^{2+} and K^+ on ALA and Chl accumulation in LA-treated cotyledons

The effect of Na^+ on ALA and Chl accumulation in LA-treated cotyledons was shown in table 6.14. In contrast to the Na^+ stimulation effect on Chl accumulation (Table 6.4), Na^+ slightly increased ALA and Chl content in LA-treated cotyledons during the light period studied. Based on the % of NaCl control, it was expected that the Na^+ stimulation effect would be 6% at 4 h and 15% at 10 h of illumination. However, it was found that Na^+ only increased ALA+Chl content by 1% and 3% at 4 and 10 h of illumination respectively. Similarly, Ca^{2+} also slightly stimulated ALA and Chl accumulation in LA-treated cotyledons during the light period studied (Table 6.15). As expected from the % of NaCl control, the Ca^{2+} stimulation effect would be 7% at

4 h and 12% at 10 h of illumination. However, Ca^{2+} only increased ALA+Chl content by 1% and 8% at 4 h and 10 h respectively. As demonstrated before, the amount of ALA accumulated was more than the decrease in Chl content due to LA administration and it appeared that not all ALA synthesized was destined for Chl formation. Consequently, the differences in degree of stimulation may be contributed by the presence of the ALA content that was not destined for Chl formation and thereby masked the stimulation effects of Na^+ and Ca^{2+} in these ALA accumulation experiments.

The effect of K^+ on ALA and Chl accumulation in LA-treated cotyledons was shown in table 6.16. K^+ stimulated ALA and Chl accumulation during the light period studied. At 4 h of illumination, K^+ increased ALA content by 47% and Chl content by 17%. The stimulation effect of K^+ on ALA+Chl content was 38%. As expected from the % of NaCl control, the K^+ stimulation effect would be 55% at 10 h of illumination. However, it was found that K^+ showed 28% increase in ALA+Chl content during a 10-h light period. The ceased increase in the K^+ stimulation effect also suggested the existence of feedback inhibition mechanism of ALA synthesizing activity by the ALA accumulated.

Effects of Na_2EGTA, verapamil and TFP on ALA and Chl accumulation in LA-treated cotyledons

Na_2EGTA, verapamil and TFP were used another approach to demonstrate the involvement of Ca^{2+} in ALA accumulation. The effect of Na_2EGTA on ALA and Chl accumulation in LA-treated cotyledons was shown in table 6.17. Na_2EGTA inhibited ALA+Chl content by about 70% during the light period studied. Verapamil decreased ALA+Chl content by about 50% at 4 h and 10 h of illumination respectively (Table 6.18). TFP showed 75% inhibition of ALA+Chl accumulation during the light period studied (Table 6.19). All these experimental evidences suggested that Ca^{2+} may regulate Chl accumulation at the level of ALA formation and probably through a calmodulin dependent mechanism.

Discussion

Measurement of ALA accumulation in LA-treated cotyledons

Levulinic acid, a competitive inhibitor of ALA dehydrase that caused ALA accumulation and inhibited Chl synthesis, is used extensively in the studies of Chl biosynthetic pathway and ALA accumulation (10, 15, 37 & 42). In the present investigation, preliminary studies on the concentrations effect of LA solution and time course accumulation of ALA and Chl in LA-treated cotyledons were performed. It was found that the amount of ALA accumulated was more than the decrease in Chl content due to LA administration (Table 6.1 and Figure 6.2). It appears that

not all of the ALA synthesized was destined for Chl formation. In fact, ALA is the universal precursor for the synthesis of Chl and other tetrapyrroles such as heme and cytochromes (4, 10 & 35). However, little is known about the proportions of the ALA content that destined for Chl formation and synthesis of other tetrapyrroles, and also the regulatory mechanisms of these ALA synthesis. Huang and Castelfranco have prostulated the presence of two distinct ALA pools in isolated developing chloroplasts: one was destined for Chl and was under phytochrome control while the other was not phytochrome regulated and was not involved in Chl synthesis (29, 30). This functional heterogenity of ALA pool has not been proven in this study. Nevertheless, it is noticed that the presence of both ALA contents for the synthesis of Chl and other tetrapyrroles may shade the regulatory effects of BA and the cations on ALA and Chl accumulation in LA-treated cotyledons (Tables 6.12-16). Another unique feature observed in concentrations effect experiment was the marked reduction in ALA content at high LA concentrations. The inhibition of ALA accumulation by high LA concentrations may be due to the side effects of LA administrated or the accumulated ALA itself. Since LA was administrated as sodium levulinate , it may be the Na^+ in the LA solution responsible for the reduction in ALA content as the same as the inhibition of

Chl accumulation by the NaCl treatment (Table 6.2). For the side effect of the accumulated ALA, ALA-induced photodynamic damage of the photosynthetic electron transport chain of cucumber cotyledons is known (69). Actually, it is not ALA itself but the compounds derived from ALA having the photodynamic properties and caused impairment of various vital cell functions. However, in the present system the conversion of ALA to those photodynamic molecules was prevented by LA. Therefore, it seems unlikely that the reduction in ALA content at high LA concentrations can be principally attributed to the accumulated ALA itself. On the other hand, a feedback inhibition mechanism of ALA synthesizing activity by the accumulated ALA was proposed.

Figure 6.2 showed the ALA accumulation curve of LA-treated cotyledons and also the greening curve of water control. ALA accumulation curve and greening curve both possessed a lag phase during the initial 2 h of illumination and a steady-state rate of ALA accumulation and Chl formation. However, when ALA content increased to 56 nmol ALA/cotyledon after 12 h of illumination, ALA accumulation slowed down. Stationary phase in greening curve was observed after 20 h of illumination. It appeared that the ALA synthesizing activity in LA-treated cotyledons was controlled through a feedback inhibition by the

accumulated ALA itself. With refer to the stimulation effects of BA and K^+ on ALA and Chl accumualtion, it was found that the stimulation effects were arrested when ALA content increased to 55-60 nmol ALA/cotyledon after 10 h of illumination (Tables 6.12, 6.13 and 6.16). All these experimental evidences suggested that there existed a feedback inhibition mechanism of the ALA synthesizing activity by the ALA accumulated. When ALA content increased to the critical quantity, supposed to be about 60 nmol ALA/cotyledon, the feedback inhibition mechanism began to operate and ALA accumulation slowed down.

Effect of BA on ALA and Chl accumulation in LA-treated cotyledons

BA is known to stimulate Chl accumulation in excised cucumber cotyledons through the enhancement of ALA synthesis (10, 15, & 42). It is believed that dark preincubation of etiolated plant materials with BA triggered the formation of ALA synthesizing system (enzymes) and thereby eliminated the lag phase of Chl formation during subsequent continuous illumination (10, 42). As reported by Lew and Tsuji, BA rapidly and specifically stimulated ALA accumulation in the dark (42). However, no BA-induced stimulation of ALA accumulation was observed at the onset of illumination (0 h) in the present study (Table 6.13). In Lew and Tsuji's study, a red light

pulse was used to eliminate the inhibition of ALA synthesis activity by Pchlide (42). It is conveniently to adopt the hypothesis that Pchlide inhibited its own formation through a feedback inhibition at the ALA formation level (10, 28). However, Huang and Castelfranco demonstrated that Pchlide did not act as a feedback inhibitor on ALA synthesis in isolated developing chloroplasts (28). Nevertheless, the red light pulse, as like preillumination effect, is known to trigger the formation of ALA synthesizing enzymes and eliminates the lag in Chl accumulation in the light (10, 16, 37, 38 & 43). Consequently, the necessarity of the red light pulse in demonstrating the BA-induced stimulation of ALA accumulation in the dark remained to be determined.

As mentioned before, the degree of stimulation and pattern of the two-fold action of BA on Chl formation are dependent on the duration of dark preincubation and the concentration of BA administrated (13, 42). Ford *et al.* reported that ALA synthesis in the light, in a manner similar to Chl synthesis activity, also varies in response to the duration of dark BA pretreatment (c.f. 42). The time course study of the BA effect on ALA and Chl accumulation in LA-treated cotyledons was shown in table 6.13. It is found that as the pattern shown in figure 4.1, the BA-stimulation pattern of ALA accumulation is also the characteristic of the duration of pretreatment and

concentration of BA used in this study although it was influenced by the 50 mM Na^+ inhibition and feedback inhibition of ALA accumulation at 10 h of illumination.

Effects of Na^+, Ca^{2+} and K^+ on ALA and Chl accumulation in LA-treated cotyledons

As demonstrated before, Na^+, Ca^{2+} and K^+ have regulatory effects on Chl accumulation in excised cucumber cotyledons. It is proposed that Ca^{2+} may act as intracellular messenger of light and hormonal signals while Na^+ and K^+ may facilitate ion transport (especially Ca^{2+}) into and out of cytoplasm and thereby regulate Chl accumulation. In the present investigation, ALA accumulation as another parameter of greening is used to examine the regulatory effects and site of action of the cations on the Chl biosynthetic pathway.

From the experimental result, the regulatory effect of K^+ is also manifested at the ALA formation level. In contrast, the stimulation effects of Na^+ and Ca^{2+} on ALA accumulation was not shown. As discussed before, the presence of both ALA contents that destined for the synthesis of Chl and other tetrapyrroles may shade the stimulation effects of Na^+ and Ca^{2+} on ALA accumulation. However, Tanaka and Tsuji reported that in the early phase of greening, Ca^{2+} stimulated ALA accumulation in the dark but inhibited it in the light (64). In this study, Ca^{2+} is

demonstrated to stimulate Chl accumulation in excised cucumber cotyledons and the stimulation effect is dependent on the amount of Ca^{2+} administrated either adjusted by concentrations or pretreatment periods (Tables 4.9 and 4.10). Therefore, stimulation of ALA accumulation by Ca^{2+} is expected although it is not significant as shown in table 6.15. It is probable that K^+, Na^+ and Ca^{2+} regulate Chl accumulation at the level of ALA formation.

Demonstration of the involvement of Ca^{2+} in ALA accumulation

Light is known to regulate chl biosynthesis at the level of ALA formation by exerting coordinated transcriptional control over the ALA synthesizing enzymes (34). As proposed before, Ca^{2+} may act as intracellular messenger of light and hormonal signals. It is supposed that light may regulate ALA synthesis through a Ca^{2+} dependent protein kinase regulatory system. Kuhlemeier *et al.* have proposed the involvement of Ca^{2+}/calmodulin dependent protein kinases in phosphorylation of specific transcriptional factor that will promote or repress gene expression in higher plants (40). The changes in cytoplasmic calcium may represent the key initial response of the cells to the light signal (5, 23 & 26). And the major consequence of such increase in cytoplasmic calcium is the activation of Ca^{2+}/calmodulin dependent protein

kinases and leading to changes to cellular processes through the phosphorylation of specific protein molecules such as transcriptional factors and enzymes (23, 26, 40, 55, 56, 57 & 71). Therefore, light may regulate ALA formation by exerting transcriptional control over the ALA synthesizing enzymes and through a Ca^{2+}/calmodulin dependent protein kinase regulatory system, and even the enzyme activity can also be modified by protein phosphorylation. Besides, BA is known to stimulate ALA formation (10, 15 & 42) and it is proposed that BA may regulate ALA accumulation by the same underlying mechanism as proposed for the light effect.

In this study, the involvement of Ca^{2+} in ALA accumulation is also demonstrated by two different approaches: preventing the changes in cytoplasmic calcium by Na_2EGTA and verapamil; and increase in cytoplasmic calcium by addition of exogeneous Ca^{2+}. As mentioned before, the stimulation effects of Ca^{2+} on ALA accumulation was not pronounced (Table 6.15). Therefore, the inhibition effects of Na_2EGTA and verapamil on ALA accumulation become the strongest evidences in supporting the involvement of Ca^{2+} in ALA accumulation. In addition, the inhibition of ALA accumulation by TFP suggested that Ca^{2+} probably regulates Chl accumulation at the level of ALA formation

and through a calmodulin dependent mechanism.

Table 6.1. Concentration effect of LA on ALA and Chl accumulation in cucumber cotyledons excised from 5-day-old plants. Cotyledons were pretreated in the dark with H_2O for 15 h and then with LA solution (pH adjusted to 6.8 with NaOH) of various concentrations for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 10 h. ALA content of 10 ml of 4% TCA (w/v) extract of 20 cotyledons was expressed as nmol ALA/cotyledon. Chl content of LA-treated cotyledons was expressed as nmol ALA equivalent, taking into account that 8 molecules of ALA were required to form 1 molecule of Chl. Mean values with SE were shown in columns. All treatments had 5 to 8 replicates.

[LA]	ALA content (nmol ALA/cotyledon)	Chl content (nmol ALA/cotyledon)
200 mM	5.94±0.00	3.20±0.32
100 mM	16.82±0.25	6.51±0.32
50 mM	45.77±0.14	9.41±0.85
20 mM	18.04±0.14	31.03±0.85
water control	0.51±0.00	43.24±0.63

Table 6.2. Effect of 50 mM NaCl on Chl accumulation in water-pretreated cotyledons. Cotyledons were incubated in the dark with H_2O for 15 h and then with 50 mM NaCl or H_2O for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 0 h, 4 h and 10 h. Chl content of 10 ml of 80% acetone extract of 10 cotyledons were expressed as ug Chl/cotyledon. Mean values with SE were shown in columns. All treatments had 4 replicates.

Treatment	Chl content (ug Chl/cotyledon)		
	Illumination time		
	0 h	4 h	10 h
H_2O(15h)+H_2O(3h)	0.10±0.01	0.95±0.04	4.46±0.11
H_2O(15h)+NaCl(3h)	0.10±0.01	0.74±0.04	3.28±0.14

Table 6.3. Effect of 50 mM NaCl on Chl accumulation in BA-pretreated and water-pretreated cotyledons. Cotyledons were incubated in the dark with $5x10^{-6}$ M BA and H_2O for 15 h and then with 50 mM NaCl or H_2O for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 0 h, 4 h and 10 h. Chl content of 10 ml of 80% acetone extract of 10 cotyledons were expressed as ug Chl/cotyledon. Mean values with SE were shown in columns. All treatments had 4 replicates.

Treatment	Chl content (ug Chl/cotyledon)		
	Illumination time		
	0 h	4 h	10 h
BA(15h)+H_2O(3h)	0.10±0.01	1.50±0.02	7.30±0.11
BA(15h)+NaCl(3h)	0.08±0.01	1.22±0.05	5.11±0.17
H_2O(15h)+H_2O(3h)	0.10±0.01	1.11±0.02	5.09±0.13
H_2O(15h)+NaCl(3h)	0.08±0.01	0.88±0.02	3.66±0.14

Table 6.4. Effect of 50 mM NaCl on Chl accumulation in NaCl-pretreated and water-pretreated cotyledons. Cotyledons were incubated in the dark with 1 mM NaCl and H_2O for 15 h and then with 50 mM NaCl or H_2O for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 4 h and 10 h. Chl content of 10 ml of 80% acetone extract of 10 cotyledons were expressed as ug Chl/cotyledon. Mean values with SE were shown in columns. All treatments had 4 replicates.

Treatment	Chl content (ug Chl/cotyledon)	
	Illumination time	
	4 h	10 h
NaCl(15h)+H_2O(3h)	1.12±0.02	5.57±0.05
NaCl(15h)+NaCl*(3h)	0.90±0.04	3.97±0.07
H_2O(15h)+H_2O(3h)	1.04±0.04	4.53±0.11
H_2O(15h)+NaCl*(3h)	0.85±0.04	3.45±0.14

Remark : the concentration of NaCl* was 50 mM.

Table 6.5. Effect of 50 mM NaCl on Chl accumulation in $CaCl_2$-pretreated and water-pretreated cotyledons. Cotyledons were incubated in the dark with 10 uM $CaCl_2$ for 9 h and H_2O for 6 h and then with 50 mM NaCl or H_2O for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 4 h and 10 h. Chl content of 10 ml of 80% acetone extract of 10 cotyledons were expressed as ug Chl/cotyledon. Mean values with SE were shown in columns. All treatments had 4 replicates.

Treatment	Chl content (ug Chl/cotyledon)	
	Illumination time	
	4 h	10 h
$CaCl_2$(9h)+H_2O(6h)+H_2O(3h)	1.14±0.05	5.57±0.07
$CaCl_2$(9h)+H_2O(6h)+NaCl(3h)	0.91±0.02	3.87±0.06
H_2O(15h)+H_2O(3h)	1.04±0.04	4.53±0.11
H_2O(15h)+NaCl(3h)	0.85±0.04	3.45±0.14

Table 6.6. Effect of 50 mM NaCl on Chl accumulation in KCl-pretreated and water-pretreated cotyledons. Cotyledons were incubated in the dark with 4 mM KCl and H_2O for 15 h and then with 50 mM NaCl or H_2O for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 0 h, 4 h and 10 h. Chl content of 10 ml of 80% acetone extract of 10 cotyledons were expressed as ug Chl/cotyledon. Mean values with SE were shown in columns. All treatments had 4 replicates.

Treatment	Chl content (ug Chl/cotyledon)		
	Illumination time		
	0 h	4 h	10 h
KCl(15h)+H_2O(3h)	0.10±0.01	1.38±0.05	8.00±0.16
KCl(15h)+NaCl(3h)	0.10±0.01	1.17±0.00	5.01±0.11
H_2O(15h)+H_2O(3h)	0.10±0.01	1.04±0.04	4.53±0.11
H_2O(15h)+NaCl(3h)	0.10±0.01	0.85±0.04	3.45±0.14

Table 6.7. Effect of 50 mM NaCl on Chl accumulation in Na_2EGTA-pretreated and water-pretreated cotyledons. Cotyledons were incubated in the dark with 4 mM Na_2EGTA and H_2O for 15 h and then with 50 mM NaCl or H_2O for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 0 h, 4 h and 10 h. Chl content of 10 ml of 80% acetone extract of 10 cotyledons were expressed as ug Chl/cotyledon. Mean values with SE were shown in columns. All treatments had 4 replicates.

Treatment	Chl content (ug Chl/cotyledon)		
	Illumination time		
	0 h	4 h	10 h
$Na_2EGTA(15h)+H_2O(3h)$	0.08±0.01	0.44±0.01	2.24±0.04
$Na_2EGTA(15h)+NaCl(3h)$	0.08±0.01	0.24±0.02	0.72±0.05
$H_2O(15h)+H_2O(3h)$	0.10±0.01	0.87±0.02	4.40±0.11
$H_2O(15h)+NaCl(3h)$	0.10±0.01	0.63±0.02	3.10±0.08

Table 6.8. Effect of 50 mM NaCl on Chl accumulation in verapamil-pretreated and water-pretreated cotyledons. Cotyledons were incubated in the dark with 100 uM verapamil and H_2O for 15 h and then with 50 mM NaCl or H_2O for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 4 h and 10 h. Chl content of 10 ml of 80% acetone extract of 10 cotyledons were expressed as ug Chl/cotyledon. Mean values with SE were shown in columns. All treatments had 4 replicates.

Treatment	Chl content (ug Chl/cotyledon)	
	Illumination time	
	4 h	10 h
verapamil(15h)+H_2O(3h)	0.63±0.02	3.02±0.06
verapamil(15h)+NaCl(3h)	0.45±0.06	1.45±0.07
H_2O(15h)+H_2O(3h)	0.91±0.04	5.04±0.16
H_2O(15h)+NaCl(3h)	0.74±0.02	3.53±0.12

Table 6.9. Effect of 50 mM NaCl on Chl accumulation in TFP-pretreated and water-pretreated cotyledons. Cotyledons were incubated in the dark with 100 uM TFP and H_2O for 15 h and then with 50 mM NaCl or H_2O for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 4 h and 10 h. Chl content of 10 ml of 80% acetone extract of 10 cotyledons were expressed as ug Chl/cotyledon. Mean values with SE were shown in columns. All treatments had 4 replicates.

Treatment	Chl content (ug Chl/cotyledon)	
	Illumination time	
	4 h	10 h
TFP(15h)+H_2O(3h)	0.46±0.02	2.52±0.12
TFP(15h)+NaCl(3h)	0.32±0.02	1.01±0.10
H_2O(15h)+H_2O(3h)	0.91±0.04	5.04±0.16
H_2O(15h)+NaCl(3h)	0.74±0.02	3.53±0.12

Table 6.10. Summary of the stimulation effects of 5 uM BA; 4 mM KCl, 10 uM $CaCl_2$ and 1 mM NaCl on Chl accumulation in 50 mM NaCl-treated cotyledons.

Treatment	% of stimulation	
	Illumination for 4 h	10 h
BA(15h)+H_2O(3h)	35%	44%
BA(15h)+NaCl(3h)*	31%	29%
KCl(15h)+H_2O(3)	33%	77%
KCl(15h)+NaCl(3)*	31%	34%
$CaCl_2$(9h)+H_2O(6h)+H_2O(3h)	10%	23%
$CaCl_2$(9h)+H_2O(6h)+NaCl(3h)*	6%	9%
$NaCl^a$(15h)+H_2O(3h)	8%	23%
$NaCl^a$(15h)+NaCl(3h)*	5%	11%

Remarks :

the concentration of $NaCl^a$ was 1 mM

* % of stimulation = $\frac{\text{Chl test solution(15h)+NaCl(3h) - Chl } H_2O\text{(15h)+NaCl(3h)}}{\text{Chl } H_2O\text{(15h)+}H_2O\text{(3h)}}$

Table 6.11. Summary of the inhibition effects of 4 mM Na_2EGTA, 100 uM verapamil and 100 uM TFP on Chl accumulation in 50 mM NaCl-treated cotyledons.

Treatment	% of inhibition	
	Illumination for 4 h	10 h
$Na_2EGTA(15h)+H_2O(3h)$	49%	49%
$Na_2EGTA(15h)+NaCl(3h)$*	45%	54%
verapamil(15h)+H_2O(3h)	30%	40%
verapamil(15h)+NaCl(3h)*	32%	41%
TFP(15h)+H_2O(3h)	49%	50%
TFP(15h)+NaCl(3h)*	46%	50%

Remarks :

* $$\%\ \text{of inhibition} = \frac{\text{Chl } H_2O(15h)+NaCl(3h) - \text{Chl test solution}(15h)+NaCl(3h)}{\text{Chl } H_2O(15h)+H_2O(3h)}$$

Table 6.12. Summary of the 50 mM NaCl effects on Chl accumulation in various treatments. Chl content was expressed as % of water control and % of NaCl control basis.

Treatment	Illumination	% of water control[a]	% of NaCl control[b]
KCl	0 h	100%	100%
	4 h	133%	138%
	10 h	177%	155%
Na_2EGTA	0 h	87%	87%
	4 h	51%	38%
	10 h	51%	23%
BA	0 h	100%	100%
	4 h	135%	139%
	10 h	144%	140%
NaCl	4 h	108%	106%
	10 h	123%	115%
$CaCl_2$	4 h	110%	107%
	10 h	123%	112%
verapamil	4 h	70%	61%
	10 h	60%	41%
TFP	4 h	51%	44%
	10 h	50%	29%

[a] % of water control = $\frac{\text{Chl content of cotyledons pretreated with test solution(15h)+}H_2O\text{(3h)}}{\text{Chl content of cotyledons pretreated with }H_2O\text{(15h)+}H_2O\text{(3h)}}$

[b] % of NaCl control = $\frac{\text{Chl content of cotyledons pretreated with test solution(15h)+NaCl(3h)}}{\text{Chl content of cotyledons pretreated with }H_2O\text{(15h)+NaCl(3h)}}$

* For $CaCl_2$ effect, cotyledons were pretreated with $CaCl_2$(9h)+H_2O(6h)+H_2O(3h) and $CaCl_2$(9h)+H_2O(6h)+NaCl(3h)

Table 6.13. Effect of BA on ALA and Chl accumulation in LA-treated cotyledons. Cotyledons were pretreated in the dark with $5x10^{-6}$ M BA and H_2O for 15 h and then with 50 mM LA solution (pH adjusted to 6.8 with NaOH) for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 0 h, 4 h and 10 h. ALA content of 10 ml of 4% TCA (w/v) extract of 20 cotyledons was expressed as nmol ALA/cotyledon. Chl content of 10 ml of 80% acetone extract of 10 cotyledons was expressed as nmol ALA equivalent, taking into account that 8 molecules of ALA were required to form 1 molecule of Chl. Mean values with SE were shown in column. All treatments had 4 to 8 replicates.

Illumination time	Treatment	ALA content (noml ALA/cotyledon)	Chl content (nmol ALA/cotyledon)	ALA+Chl content (nmol ALA/cotyledon)
0 h	BA(15h)+LA(3h)	0.36±0.00	0.53±0.04	0.90
	H_2O(15h)+LA(3h)	0.36±0.00	0.53±0.04	0.90
4 h	BA(15h)+LA(3h)	14.00±0.13	3.04±0.04	17.03
	H_2O(15h)+LA(3h)	10.00±0.13	2.34±0.22	12.34
10 h	BA(15h)+LA(3h)	60.32±0.08	10.63±0.86	70.85
	H_2O(15h)+LA(3h)	47.46±0.08	7.46±0.42	54.92

Table 6.14. Effect of NaCl on ALA and Chl accumulation in LA-treated cotyledons. Cotyledons were pretreated in the dark with 1 mM NaCl and H_2O for 15 h and then with 50 mM LA solution (pH adjusted to 6.8 with NaOH) for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 4 h and 10 h. ALA and Chl contents were expressed as nmol ALA/cotyledon and other notes were as in the legand to table 6.15.

Illumination time	Treatment	ALA content (noml ALA/cotyledon)	Chl content (nmol ALA/cotyledon)	ALA+Chl content (nmol ALA/cotyledon)
4 h	NaCl(15h)+LA(3h)	9.94±0.25	2.80±0.42	12.74
	H_2O(15h)+LA(3h)	9.99±0.44	2.66±0.22	12.65
10 h	NaCl(15h)+LA(3h)	47.34±0.48	9.17±0.42	56.51
	H_2O(15h)+LA(3h)	45.71±0.13	9.06±0.32	54.77

Table 6.15. Effect of $CaCl_2$ on ALA and Chl accumulation in LA-treated cotyledons. Cotyledons were pretreated in the dark with 10 uM $CaCl_2$ for 9h and H_2O for 6h and then with 50 mM LA solution (pH adjusted to 6.8 with NaOH) for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 4 h and 10 h. ALA and Chl contents were expressed as nmol ALA/cotyledon and other notes were as in the legand to table 6.13.

Illumination time	Treatment	ALA content (noml ALA/cotyledon)	Chl content (nmol ALA/cotyledon)	ALA+Chl content (nmol ALA/cotyledon)
4 h	$CaCl_2$(9h)+H_2O(6h)+LA(3h)	10.02±0.25	2.62±0.10	12.68
	H_2O(15h)+LA(3h)	9.99±0.44	2.66±0.22	12.65
10 h	$CaCl_2$(9h)+H_2O(6h)+LA(3h)	49.62±0.75	9.70±0.86	59.33
	H_2O(15h)+LA(3h)	45.71±0.13	9.06±0.32	54.77

Table 6.16. Effect of KCl on ALA and Chl accumulation in LA-treated cotyledons. Cotyledons were pretreated in the dark with 4 mM KCl and H_2O for 15 h and then with 50 mM LA solution (pH adjusted to 6.8 with NaOH) for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 0 h, 4 h and 10 h. ALA and Chl content were expressed as nmol ALA/cotyledon and other notes were as in the legand to table 6.13.

Illumination time	Treatment	ALA content (noml ALA/cotyledon)	Chl content (nmol ALA/cotyledon)	ALA+Chl content (nmol ALA/cotyledon)
0 h	KCl(15h)+LA(3h)	0.42±0.00	0.53±0.04	0.95
	H_2O(15h)+LA(3h)	0.42±0.00	0.53±0.04	0.95
4 h	KCl(15h)+LA(3h)	11.95±0.15	4.38±0.22	16.32
	H_2O(15h)+LA(3h)	8.10±0.05	3.74±0.32	11.84
10 h	KCl(15h)+LA(3h)	55.06±0.36	14.93±0.86	69.99
	H_2O(15h)+LA(3h)	44.00±0.10	10.56±0.54	54.56

Table 6.17. Effect of Na_2EGTA on ALA and Chl accumulation in LA-treated cotyledons. Cotyledons were pretreated in the dark with 4 mM Na_2EGTA and H_2O for 15 h and then with 50 mM LA solution (pH adjusted to 6.8 with NaOH) for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 0 h, 4 h and 10 h. ALA and Chl contents were expressed as nmol ALA/cotyledon and other notes were as in the legand to table 6.13.

Illumination time	Treatment	ALA content (noml ALA/cotyledon)	Chl content (nmol ALA/cotyledon)	ALA+Chl content (nmol ALA/cotyledon)
0 h	Na_2EGTA(15h)+LA(3h)	0.38±0.00	0.53±0.04	0.91
	H_2O(15h)+LA(3h)	0.38±0.00	0.53±0.04	0.91
4 h	Na_2EGTA(15h)+LA(3h)	2.76±0.00	1.18±0.10	3.95
	H_2O(15h)+LA(3h)	10.04±0.07	3.74±0.32	13.77
10 h	Na_2EGTA(15h)+LA(3h)	12.76±0.00	3.19±0.64	15.96
	H_2O(15h)+LA(3h)	45.91±0.14	10.56±0.54	56.47

Table 6.18. Effect of verapamil on ALA and Chl accumulation in LA-treated cotyledons. Cotyledons were pretreated in the dark with 100 uM verapamil and H_2O for 15 h and then with 50 mM LA solution (pH adjusted to 6.8 with NaOH) for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 4 h and 10 h. ALA and Chl contents were expressed as nmol ALA/cotyledon and other notes were as in the legand to table 6.13.

Illumination time	Treatment	ALA content (noml ALA/cotyledon)	Chl content (nmol ALA/cotyledon)	ALA+Chl content (nmol ALA/cotyledon)
4 h	verapamil(15h)+LA(3h)	4.55±0.03	1.28±0.10	5.83
	H_2O(15h)+LA(3h)	9.99±0.44	2.66±0.22	12.65
10 h	verapamil(15h)+LA(3h)	21.91±0.02	5.54±0.32	27.45
	H_2O(15h)+LA(3h)	45.71±0.13	9.06±0.32	54.77

Table 6.19. Effect of TFP on ALA and Chl accumulation in LA-treated cotyledons. Cotyledons were pretreated in the dark with 100 uM TFP and H_2O for 15 h and then with 50 mM LA solution (pH adjusted to 6.8 with NaOH) for 3 h. Afterwards, cotyledons were exposed to light (50 $uEm^{-2}s^{-1}$ PAR) for 4 h and 10 h. ALA and Chl contents were expressed as nmol ALA/cotyledon and other notes were as in the legand to table 6.13.

Illumination time	Treatment	ALA content (noml ALA/cotyledon)	Chl content (nmol ALA/cotyledon)	ALA+Chl content (nmol ALA/cotyledon)
4 h	TFP(15h)+LA(3h)	2.35±0.04	0.74±0.04	3.10
	H_2O(15h)+LA(3h)	9.99±0.44	2.66±0.22	12.65
10 h	TFP(15h)+LA(3h)	11.31±0.13	2.85±0.10	14.24
	H_2O(15h)+LA(3h)	45.71±0.13	9.06±0.32	54.77

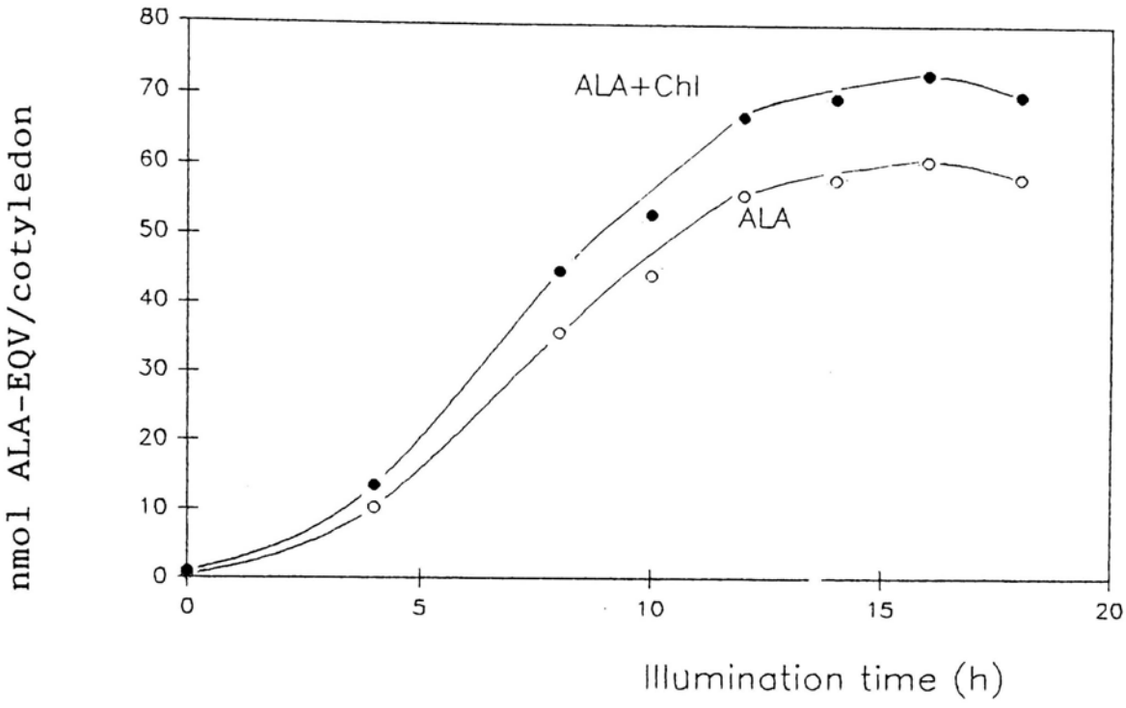

Figure 6.1. Time course of ALA and Chl accumulation under continuous illumination in LA-treated cotyledons. (o—o) represented only the ALA content; (●—●) represented the ALA+Chl content. Chl content was expressed as nmol ALA equivalent/cotyledon taking into account that 8 molecules of ALA were required to form 1 molecule of Chl. Each point represented the mean of 4 replicate samples.

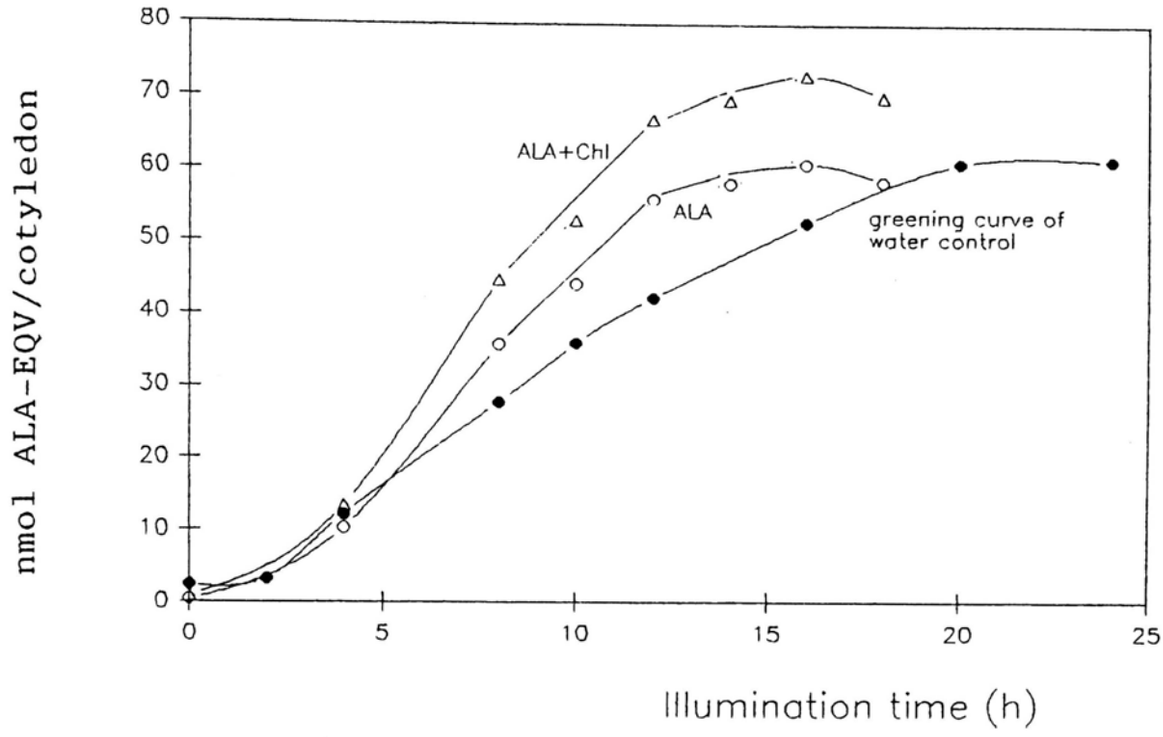

Figure 6.2. ALA accumulation curve of LA-treated cotyledon and greening curve of water control.(o—o) represented the ALA content and (Δ—Δ) represented the ALA+Chl content in LA-treated cotyledons. (●—●) represented the Chl content of water control. Other notes were as in figure 6.1.

Chapter 7

Regulation of 5-Aminolevulinic Acid Accumulation in Isolated Developing Chloroplasts

Introduction

Greening bioassay in isolated developing chloroplasts is also frequently employed for the studies of Chl biosynthetic pathway and its regulation (10, 24, 28, 29, 30, 31 & 54). As the developing chloroplasts are isolated from greening cotyledons, they have already possessed the precursors of nuclear and chloroplast origins for Chl biosynthesis. Consequently, only feeding of appropriate substrates, cofactors and inhibitors or blockers of the synthetic pathway, any parameters of greening can be monitored by this *in organello* system.

In the present investigation, the regulatory effects of BA and the cations on ALA accumulation are examined in isolated developing chloroplasts. Since greening bioassay in excised cotyledons includes the involvement of nucleus, cytoplasm and chloroplasts in Chl synthesis, it is not known where are the subcellular site of action of BA and the cations. In contrast, greening bioassay in isolated developing chloroplasts excludes the influences of cytoplasm and nucleus, it allows direct and more conclusive investigation of the regulatory effects of BA and the

cations on Chl formation. Therefore, the aims of this study are to determine the subcellular site of action and propose the possible modes of action of BA and the cations on greening process.

Materials and Methods

Materials

Cucumber seeds (*Cucumis sativus* L. cv. Sure Green) were obtained from Known You Seed Co.,Ltd., Taiwan.

The following chemicals are purchased from Sigma: ATP, BA, BSA, $CaCl_2.2H_2O$, $CuSO_4.5H_2O$, cysteine, DMAB, EGTA, glucose-6-phosphate, L-glutamic acid, Hepes, KCl, LA, Na_2EDTA, NADPH, Tes, TFP and verapamil. Na_2CO_3, Na K-tartrate, NaOH and sodium acetate were obtained from Riedel-de Haën. Sorbitol was purchased from BDH Chemical Ltd, NaCl was from Anala R, Folin-Ciocalteu's phenol reagent, glacial acetic aicd and $MgCl_2.6H_2O$ were from Merck. Trichloroacetic acid was obtained from Ajax Chemicals and acetylaectone was from Peking Chemical Work. Percoll was obtained from Pharmacia.

Methods

Plant materials

The selection of suitable plant materials for chloroplast isolation was based on the time course study of

Chl accumulation in intact greening cotyledons of different ages. Cucumber seeds were soaked in distilled water for 15 min before planted in vermiculite which had been watered once with 250 ml distilled water. Seeds were germinated in the dark at 28°C. After germination for 3 days, 4 days and 5 days, etiolated seedlings were exposed to continuous illumination under the light intensity at 80uEm^{-2}s^{-1} PAR. After various periods of illumination, cotyledons were harvested and blotted. Chl of ten cotyledons was extracted with 10 ml of 80% acetone and the homogenate was centrifuged at 3000 *g* for 15 min. Chl content in the supernatant was determined by measuring absorbance at 663 nm and was expressed as ug Chl/cotyledon.

Chloroplast isolation

Cucumber seeds were germinated in the dark at 28°C for 3 days. Afterwards, etiolated seedlings were illuminated (at 80 uEm^{-2}s^{-1} PAR) for 34 h and then the greening cotyledons were harvested. Chloroplast isolation was mainly based on the procedure described by Fufsler *et al.* (24, 54). For a typical isolation starting with 60 gm of plant tissue, the greening cotyledons were ground gently in mortar and pestle with 125 ml of grinding buffer (0.5 M sorbitol, 10 mM Hepes, 20 mM Tes, 1 mM EDTA, 1 mM $MgCl_2$, 5 mM Cys and 0.2% BSA, pH 7.7). The homogenate was then

filtered through four layers of miracloth and the filtrate was centrifuged at 10000 *g* for 1 min. Pellet was collected and was resuspended in 10 ml of grinding buffer. The suspension was then centrifuged at 150 *g* for 1 min. Afterwards, supernatant was collected and was centrifuged at 2000 *g* for 2 min. The pellet obtained was resuspended in 10 ml of resuspension buffer (grinding buffer without BSA). Five ml of the suspension was then layered over 35 ml of 45% Percoll in the identical resuspension buffer, contained in a 50 ml centrifuge tubes and usually two such tubes were used. The tubes were centrifuged at 6000 *g* for 5 min in a Sorvall RC-5 centrifuge equipped with a HB-4 rotor or in a Beckman J2-M1 centrifuge equipped with JA-21 rotor. The small pellets consisting of essentially intact plastids were collected and were resuspended gently in 5 ml of 2x incubation buffer (1 M sorbitol, 20 mM Hepes, 40 mM Tes, 2 mM EDTA, 2 mM $MgCl_2$ and 20 mM LVA, pH 7.7).

Incubation conditions

In ALA accumulation experiments, incubations of isolated developing chloroplasts were carried out in 25 ml Erlenmyer flasks in 1 ml total volume consisting of 0.5 ml of 2x incubation buffer containing 500 to 1000 ug plastid protein as determined by the Lowry method (44), 0.25 ml of test solution or distilled water and 0.25 ml of 4x

substrate-cofactor mixture (24 mM Glu, 12 mM ATP, 16 mM NADPH and 16 mM glucose-6-phosphate). Incubations were carried out at 28°C either in the light ($50uEm^{-2}s^{-1}$ PAR) or in the dark with the Erlenmyer flasks kept in orbital shaking at 50 rpm. The reactions were initiated by the addition of 4x substrate-cofactor mixture and were terminated by the addition of 0.2 ml of 24% (w/v) trichloroacetic acid.

ALA assay

The ALA content in 1 ml incubation mixture was extracted with 0.2 ml of 24% (w/v) trichloroacetic acid. The homogenate was centrifuged at 13000 rpm for 5 min by a MSE microcentrifuge. And then, 1 ml supernatant (ALA extract) was added to 0.47 ml of 1 M sodium acetate and 0.03 ml of acetylacetone. The mixture was heated at 100°C for 15 min. Afterwards, equal aliquots of the sample and modified Ehrlich's reagent (1 gm of DMAB in 10 ml of 70% perchloric acid and 40 ml of glacial acetic acid) were mixed (45). Fifteen min later, the ALA content in the reaction mixture was determined by measuring absorbance at 554 nm against a control which had been treated identically but where acetylacetone was replaced by distilled water. The ALA content which expressed as nmol ALA/mg PROTEIN was calculated from a standard curve with samples containing

known amounts of ALA.

Measurement of ALA accumulation in LA-treated isolated developing chloroplasts

Developing chloroplasts were isolated from greening cotyledons as previously described. Incubations of isolated chloroplasts were carried out in 25 ml Erlenmyer flasks in 1 ml total volume consisting of 0.5 ml of chloroplast suspension in 2x incubation buffer without LA, 0.25 ml distilled water or LA of final concentrations at 10, 15, 20 and 22.5 mM, and 0.25 ml of 4x substrate-cofactor mixture. Incubations were carried out in the light (50 $uEm^{-2}s^{-1}$ PAR) for 2 h. The ALA content in the incubation mixtures was then determined.

Measurement of ALA accumulation in BA-, cations-, Na_2EGTA, verapamil- and TFP- treated isolated developing chloroplasts

Developing chloroplasts were isolated as previously described. Incubations of isolated chloroplasts were carried out in 25 ml Erlenmyer flasks in 1 ml total volume consisting of 0.5 ml of chloroplast suspension in 2x incubation buffer, 0.25 ml of test solutions or distilled water, and 0.25 ml of 4x substrate-cofactor mixture. Incubations were carried out in the light (50 $uEm^{-2}s^{-1}$ PAR) for 2 h. The ALA content in the incubation mixtures was then determined. The test solutions in this study included

BA, NaCl, $CaCl_2$, KCl, Na_2EGTA, verapamil and TFP of various concentrations.

Results

Time course study of Chl accumulation in intact greening cotyledons of different ages

As mentioned before, the selection of suitable plant materials for chloroplast isolation was based on the Chl synthesizing activity in intact greening cotyledons. It was assumed that developing chloroplasts isolated from greening cotyledons having a higher Chl synthesizing activity would be more active and ideal for *in organello* assay.

Figure 7.1 showed the greening curves of intact etiolated cotyledons of different ages. Among the ages of cotyledons studied, 3-day-old cotyledons showed the highest Chl synthesizing activity during the light period studied. The Chl content increased slowly during the initial 20 h of illumination and then an exponential increase in Chl content was observed. For the 4-day-old and 5-day-old cotyledons, Chl content also accumulated in a manner similar to that in 3-day-old cotyledons with a long lag phase and an exponential increase in Chl accumulation afterwards. However, the Chl content in these cotyledons were much less than that in 3-day-old cotyledons. Consequently, 3-day-old cotyledons after illumination for

28 h and 34 h were harvested and developing chloroplasts were isolated. Preliminary study showed that ALA synthesizing activity was too low in developing chloroplasts isolated from 28 h illuminated cotyledons. In contrast, the developing chloroplasts isolated from 34 h illuminated cotyledons showed a considerable ALA synthesizing activity and were employed for the ALA accumulation experiments.

Measurement of ALA synthesizing activity in isolated developing chloroplasts incubated in the dark and light

Figure 7.2 showed the ALA synthesizing activity in isolated developing chloroplasts incubated in the light and in the dark. It was observed that the ALA synthesizing activity was more pronounced when isolated chloroplasts were incubated in the light. The ALA content was 4 nmol ALA/mg protein at the onset of illumination (0 h) and increased to 11 nmol ALA/mg protein during a 2-h light period. In contrast, the ALA content was only 5 nmol ALA/mg protein after a 2-h dark incubation. It appears that light may regulate ALA synthesis by exerting not only transcriptional control over the ALA synthesizing enzymes but also control of the enzyme activities.

Concentrations effect of LA on ALA accumulation in isolated developing chloroplasts

The concentrations effect of LA on ALA accumulation in isolated developing chloroplasts was shown in table 7.1. Only a trace amount of ALA was accumulated in the absence of LA (control). LA caused ALA accumulation. The ALA content in the isolated chloroplasts was about 11 to 12 nmol ALA/mg protein and was independent on the concentrations of LA administrated. 10 mM LA was used for the ALA accumulation experiments.

Effects of BA and the cations on ALA accumulation in isolated developing chloroplasts

Table 7.2 showed the effect of BA on ALA accumulation in isolated developing chloroplasts. Among the BA concentrations tested, 0.1 uM BA increased ALA content by 2% and 1 uM BA stimulated ALA accumulation by 5%. 10 uM BA showed 1% increase in ALA content. The pronounced BA stimulation effect was not shown in this *in organello* assay.

For the cations, Na^+ showed no stimulation effect on ALA accumulation in isolated chloroplasts (Table 7.3). Ca^{2+} of concentrations ranged from 1 uM to 1 mM increased ALA accumulation by 7% but the stimulation effect was decreased to 1 % by 2.5 mM Ca^{2+} (Table 7.4). K^+ also showed no significant stimulation effect on ALA accumulation among the concentrations tested (Table 7.5). In contrast to the stimulation effects demonstrated in excised cotyledons, BA

and the cations showed no pronounced stimulation effects, or even no effects on ALA accumulation in isolated developing chloroplasts.

Effects of Na_2EGTA, verapamil and TFP on ALA accumulation in isolated developing chloroplasts

Na_2EGTA, verapamil and TFP were demonstrated to inhibit Chl and ALA accumulation in excised cotyledons. However, their inhibition effects on ALA accumulation were not shown in isolated developing chloroplasts. Table 7.6 showed the effect of Na_2EGTA on ALA accumulation in isolated developing chloroplasts. Na_2EGTA at 0.5 mM or higher showed 3 to 6% inhibition of ALA accumulation but the inihbition effect was considered to be not significant according to statistical analysis. Verapamil also showed no inhibition effect on ALA accumulation (Table 7.7). On the other hand, insignificant stimulation of ALA accumulation by verapamil at 100 uM and 1000 uM was observed. The effect of TFP on ALA accumulation in isolated chloroplasts was shown in table 7.8. TFP also showed no significant inhibition effect on ALA accumulation among the concentrations tested.

Discussion

Light regulation of ALA synthesizing activity in isolated developing chloroplasts

In higher plants and green algae, ALA is synthesized from glutamate of Glu-tRNA via the two step five carbon pathway which involving Glu-tRNA reductase (GluTR) and glutamate-1-semialdehyde 2,1-aminomutase (GSA-AM) (10, 34 & 35). The glutamate transfer RNA ($tRNA^{Glu}$) is chloroplast encoded, whereas the genes for GluTR and GSA-AM are believed to be nuclear encoded (34, 35). Light is known to regulate ALA formation by exerting transcriptional control over the enzymes of five carbon pathway (34).

As the developing chloroplasts are isolated from greening cotyledons, they have already possessed the precursors of nuclear and chloroplast origins for ALA synthesis. Therefore, ALA accumulation experiments in isolated developing chloroplasts are frequently carried out in the dark with exogeneous cofactors such as ATP and reducing power. It is presumed that the function of incubation under photosynthetic condition is to provide the isolated chloroplasts with more ATP and reducing power for ALA synthesis. However, in the present study it was found that ALA accumulation in isolated chloroplasts was light-dependent. The ALA synthesizing activity was more pronounced only when incubation was carried out in the light (Figure 7.2). As proposed before, light may regulate ALA synthesis at transcriptional level through a Ca^{2+} and calmodulin dependent protein phosphorylation mechanism. It

is not surprising even the ALA synthesizing enzymes activities can also be modulated by protein phosphorylation. Although the available evidences concerning the light regulation of ALA synthesizing enzymes activities in higher plants are very limited (29), it is supposed that light may regulate ALA synthesis by exerting not only transcriptional control over the enzymes of the five carbon pathway but also control of the enzyme activity.

Regulation of ALA accumulation in isolated developing chloroplasts

In the present study, the regulatory effects of BA and the cations on ALA accumulation were examined in isolated developing chloroplasts. A wide range of concentrations of BA, Na^+, Ca^{2+} and K^+ as well as Na_2EGTA, verapamil and TFP was tested. Those concentrations included the ones showing pronounced stimulation and inhibition effects on Chl and ALA accumulation in excised cotyledons. This ensured that the stimulation and inhibition effects of the test solutions would be also manifested in this *in organello* assay once if their subcellular sites of action were in the chloroplasts.

As shown in the experimental results, the stimulation effects of BA, Na^+, Ca^{2+} and K^+, and the inhibition effects of Na_2EGTA, verapamil and TFP on ALA accumulation were not manifested in isolated developing chloroplasts. It appears

that chloroplasts are not the subcellular site of action of BA and the cations. Arnold and Fletcher reported that protein synthesis in the cytoplasm are essential for the stimulation of greening by BA and K^+ (2). In addition, RNA synthesis was also required for the BA effect (2). In fact, BA is known to stimulate Chl accumulation through the enhancement of ALA synthesis and promotion of *cab* gene expression (10, 15, 42 & 53). It is probable that BA regulates greening process by involving nuclear and cytoplasmic events: transcriptions in nucleus and protein synthesis in cytoplasm.

For the cations, Ca^{2+} is proposed to be the intracellular messenger of light and hormonal signals and regulate Chl synthesis through a calmodulin dependent protein phosphorylation mechanism. Indeed, protein phosphorylation is now emerging as the major mechanism by which Ca^{2+} and calmodulin regulate biochemical events inside the cells in response to cellular or environmental signals (71). Through the phosphorylation of specific protein molecules such as enzymes, functional and structural membrane proteins and transcriptional factors, a diverse array of physiological phenomena are tightly regulated (40, 57). Consequently, Ca^{2+} may exert its regulatory effects in the cytoplasm for enzyme protein phosphorylation (52), in the nucleus for phosphorylation of

transcriptional factors (40), and even in the chloroplasts for phosphorylation of envelope proteins and also thylakoid and stromal proteins (6). However, in this study Ca^{2+} only slightly stimulated ALA accumulation in isolated developing chloroplasts (Table 7.4). K^+, as discussed before, exerted its regulatory effect essentially in the cytoplasm (2). However, the mechanism of K^+-induced stimulation of greening is not known. In addition, the effect of Na^+ on stimulation of Chl and ALA accumulation have not been mentioned.

The present results suggest that chloroplasts are not the subcellular site of action of BA and the cations. Therefore, the possibility of the cations acting as enzyme cofactors or involving in stabilization of pigment-protein complexes in greening process can be excluded. It is proposed that BA and the cations may mediate their effects on ALA and Chl formation through promotion or repression of gene expression in nucleus, regulation of protein synthesis and modulation of enzyme activity in cytoplasm and also chloroplast, and even regulation of transportation of nuclear gene products into chloroplasts.

Table 7.1. Effect of LA concentrations on ALA accumulation in developing chloroplasts isolated from greening seedlings. Isolated chloroplasts were incubated with 500 mM sorbitol, 10 mM Hepes, 20 mM Tes, 1 mM EDTA and 1 mM $MgCl_2$, 6 mM Glu, 3 mM ATP, 4 mM NADPH, 4 mM glucose-6-phosphate, and LA of different concentrations. Incubations were carried out in the light (50 $uEm^{-2}s^{-1}$ PAR) for 2 h. ALA content was expressed as nmol ALA/mg PROTEIN. Mean values with SD were shown in column, and means within column followed by the same letter are not significant different at P=0.05 according to Student-Newman-Keuls test. All treatments were in duplicate.

[LA]	ALA content (nmol ALA/mg PROTEIN)
22.5 mM	10.94±0.09*a*
20 mM	12.16±1.20*a*
15 mM	12.65±0.00*a*
10 mM	11.88±0.37*a*
0 mM (control)	3.70±0.09*b*

Table 7.2. Effect of BA on ALA accumulation in developing chloroplasts isolated from greening seedlings. Isolated chloroplasts were incubated with BA of different concentrations and other additions as indicated in 'Materials and Methods'. Incubations were carried out in the light (50 $uEm^{-2}s^{-1}$ PAR) for 2 h. ALA content was expressed as nmol ALA/mg PROTEIN. Other notes were as in table 7.1.

[BA]	ALA content (nmol ALA/mg PROTEIN)
10 uM	10.92±0.00*a*
1 uM	11.33±0.12*a*
0.1 uM	11.07±0.48*a*
water control	10.82±0.00*a*

Table 7.3. Effect of NaCl on ALA accumulation in developing chloroplasts isolated from greening seedlings. Isolated chloroplasts were incubated NaCl of different concentrations and other additions as indicated in 'Materials and Methods'. Incubations were carried out in the light (50 $uEm^{-2}s^{-1}$ PAR) for 2 h. ALA content was expressed as nmol ALA/mg PROTEIN. Other notes were as in table 7.1.

[NaCl]	ALA content (nmol ALA/mg PROTEIN)
1000 uM	12.60±0.00*a*
100 uM	12.50±0.32*a*
10 uM	12.47±0.16*a*
water control	12.78±0.00*a*

Table 7.4. Effect of $CaCl_2$ on ALA accumulation in developing chloroplasts isolated from greening seedlings. Isolated chloroplasts were incubated with $CaCl_2$ of different concentrations and other additions as indicated in 'Materials and Methods'. Incubations were carried out in the light (50 $uEm^{-2}s^{-1}$ PAR) for 2 h. ALA content was expressed as nmol ALA/mg PROTEIN. Other notes were as in table 7.1.

$[CaCl_2]$	ALA content (nmol ALA/mg PROTEIN)
2500 uM	11.03±0.12*a*
1000 uM	11.68±0.18*a*
100 uM	11.68±0.00*a*
10 uM	11.58±0.43*a*
1 uM	11.58±0.31*a*
water control	10.92±0.12*a*

Table 7.5. Effect of KCl on ALA accumulation in developing chloroplasts isolated from greening seedlings. Isolated chloroplasts were incubated with KCl of different concentrations and other additions as indicated in 'Materials and Methods'. Incubations were carried out in the light (50 $uEm^{-2}s^{-1}$ PAR) for 2 h. ALA content was expressed as nmol ALA/mg PROTEIN. Other notes were as in table 7.1.

[KCl]	ALA content (nmol ALA/mg PROTEIN)
5000 uM	10.90±0.00*a*
1000 uM	10.84±0.12*a*
500 uM	10.96±0.48*a*
water control	10.67±0.00*a*

Table 7.6. Effect of Na_2EGTA on ALA accumulation in developing chloroplasts isolated from greening seedlings. Isolated chloroplasts were incubated with Na_2EGTA of different concentrations and other additions as indicated in 'Materials and Methods'. Incubations were carried out in the light (50 $uEm^{-2}s^{-1}$ PAR) for 2 h. ALA content was expressed as nmol ALA/mg PROTEIN. Other notes were as in table 7.1.

[Na_2EGTA]	ALA content (nmol ALA/mg PROTEIN)
2000 uM	10.42±0.06*a*
1000 uM	10.70±0.38*a*
500 uM	10.84±0.00*a*
50 uM	11.09±0.00*a*
5 uM	11.21±0.65*a*
water control	11.12±0.06*a*

Table 7.7. Effect of verapamil on ALA accumulation in developing chloroplasts isolated from greening seedlings. Isolated chloroplasts were incubated with verapamil of different concentrations and other additions as indicated in 'Materials and Methods'. Incubations were carried out in the light (50 $uEm^{-2}s^{-1}$ PAR) for 2 h. ALA content was expressed as nmol ALA/mg PROTEIN. Other notes were as in table 7.1.

[verapamil]	ALA content (nmol ALA/mg PROTEIN)
1000 uM	11.42±0.00*a*
100 uM	11.42±0.17*a*
10 uM	10.83±0.00*a*
water control	10.71±0.00*a*

Table 7.8. Effect of TFP on ALA accumulation in developing chloroplasts isolated from greening seedlings. Isolated chloroplasts were incubated with TFP of different concentrations and other additions as indicated in 'Materials and Methods'. Incubations were carried out in the light (50 $uEm^{-2}s^{-1}$ PAR) for 2 h. ALA content was expressed as nmol ALA/mg PROTEIN. Other notes were as in table 7.1.

[TFP]	ALA content (nmol ALA/mg PROTEIN)
1000 uM	11.87±0.83*a*
100 uM	12.76±0.35*a*
10 uM	12.31±0.35*a*
water control	12.50±0.39*a*

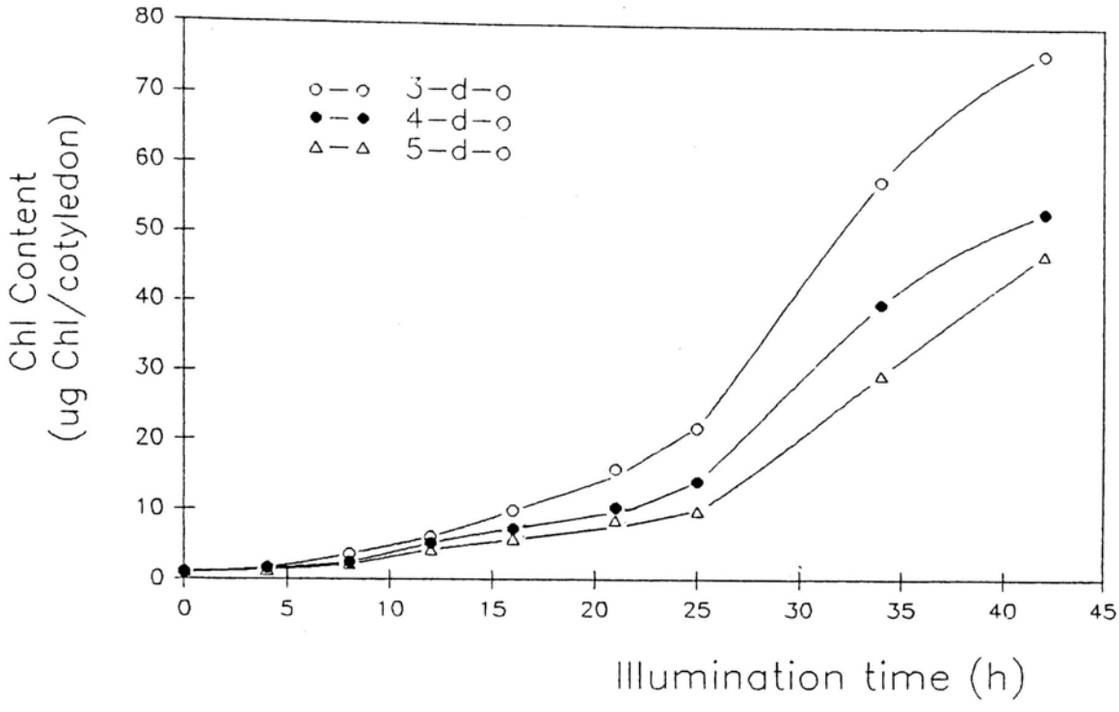

Figure 7.1. Time course of Chl accumulation under continuous illumination in intact etiolated cotyledons of different ages. Cucumber seeds were dark germinated for 3 d, 4 d and 5 d. Afterwards, the etiolated seedlings were illuminated at 80 $uEm^{-2}s^{-1}$ PAR for various periods. Chl content of 10 ml of 80% acetone extract of ten cotyledons was expressed as ug Chl/cotyledon. Each point represented the mean of 8 replicate samples.

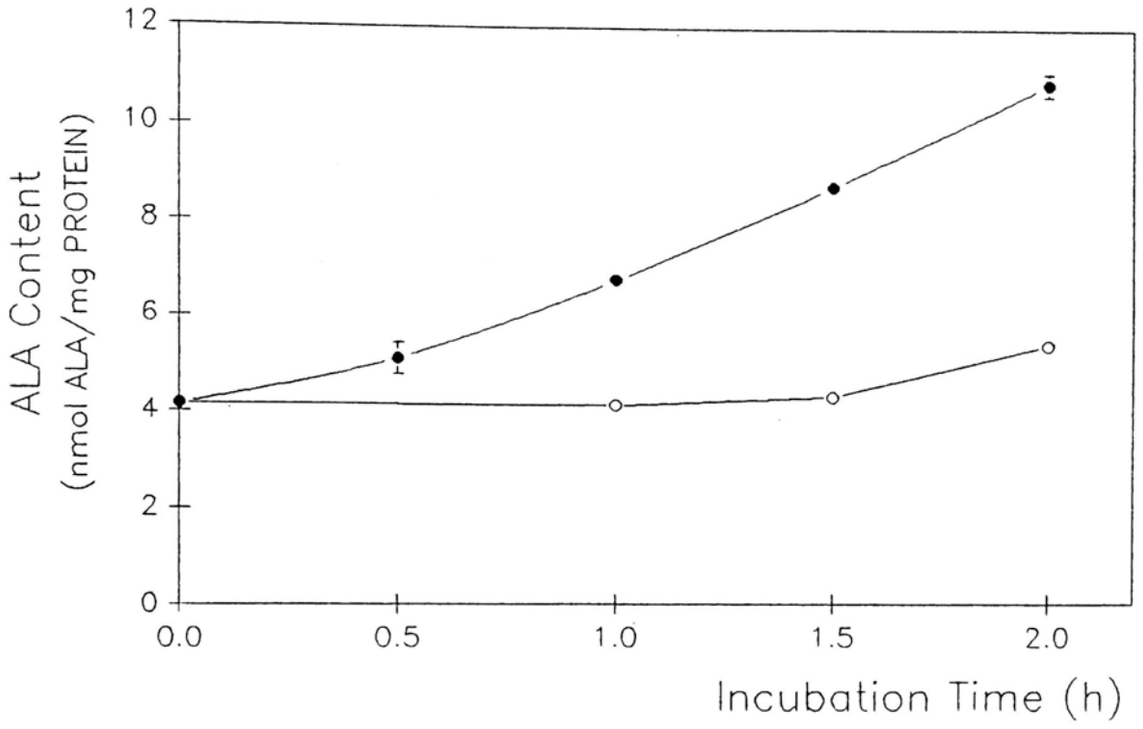

Figure 7.2. ALA synthesizing activity in isolated developing chloroplasts incubated in the light (●—●) and in the dark (o—o). Cucumber seeds were dark germinated for 3 d. After 34 h of illumination (80 $uEm^{-2}s^{-1}$ PAR), the cotyledons were harvested and developing chloroplasts were isolated. Isolated developing chloroplasts were incubated either in the dark or in the light (50 $uEm^{-2}s^{-1}$ PAR) with the additions indicated in 'Materials and Methods'. Each point represented duplicate samples.

Chapter 8

Conclusion

In the present study, attempts have been made to explore the possible modes of action of BA and certain cations on greening process. With refer to the greening bioassay in excised cotyledons, K^+ was demonstrated to stimulate Chl accumulation and the existence of a threshold level of K^+ for maximum stimulation is proposed. Na^+ also showed stimulation effect on Chl accumulation. The Na^+ stimulation effect appears to be independent on the duration of pretreatment period and also the concentration administrated. It is supposed that Na^+ may also possess a very low threshold level for maximum stimulation of Chl accumulation. However, 50 mM Na^+, in contrast to the previous studies (15), showed inhibition effect on Chl accumulation (Table 6.2). It is surprising about the discrepancy effects of Na^+ on Chl accumulation. Ca^{2+} was also demonstrated to stimulate Chl accumulation but the degree of stimulation was dependent on the amount of Ca^{2+} administrated. In contrast to the previous reports (64), low concentrations of Ca^{2+} (at uM level) and shortened pretreatment period showed pronounced stimulation effect on Chl accumulation. In fact, we realize that it is difficult to mimic the natural increase in cytoplasmic Ca^{2+} by

addition of exogeneous Ca^{2+} or Ca ionophore. As very little is known about the magnitude, duration, speed and spatial organization of the changes in cytoplasmic Ca^{2+}, the uncontrolled generation of increases in cytoplasmic Ca^{2+} is often inhibitory. Consequently, preventing the changes in cytoplasmic Ca^{2+} by Na_2EGTA and verapamil were used as another experimental approach to demonstrate the involvement of Ca^{2+} in greening process. It was found that Chl accumulation was inhibited by Na_2EGTA and verapamil treatments. In addition, inhibition of Chl acumulation by TFP also suggests that Ca^{2+} probably regulates Chl accumulation through a calmodulin dependent mechanism. It is proposed that Ca^{2+} may act as intracellular messenger of light signal and regulates Chl accumulation through a Ca^{2+} and calmodulin activated protein kinase regulatory system. For K^+ and Na^+, they are proposed to be involved in facilitating transport of ions (especially Ca^{2+}) into and out of cytoplasm and thereby regulate Chl accumulation.

BA was demonstrated to stimulate Chl accumulation in excised cotyledons. Treatment of BA-treated cotyledons with Na_2EGTA and verapamil abolished the BA stimulation effect. Moreover, application of TFP to BA-treated cotyledons also resulted in inhibition of Chl synthesis. In sequence experiments as well, the BA-induced stimulation effect was inhibited by Na_2EGTA, verapamil and TFP treatments.

However, it is interesting to note that treatment of verapamil-pretreated cotyledons with BA reversed the verapamil inhibition effect. It appears that there existed the antagonism between BA and verapamil; BA may induce an increase in cytoplasmic Ca^{2+} whereas verapamil inhibits it. Moreover, the TFP inhibition effect also seemed to be partially reversed by the BA treatment. Consequently, it is suggested that Ca^{2+} may be involved in BA-induced stimulation of Chl accumulation in excised cucumber cotyledons and probably through a calmodulin dependent mechanism. In addition, Ca^{2+} is proposed to be the intracellular messenger of cytokinin signal.

In ALA accumulation experiments, the regulatory effects of BA and the cations were also manifested in excised cotyledons but they were not shown in isolated developing chloroplasts. It appears that BA and the cations regulate Chl biosynthesis at the level of ALA formation and their subcellular site of action is probably not in chloroplasts. As proposed before, Ca^{2+} may act as intracellular messenger of light and cytokinin signals. Light is known to regulate ALA and thereby Chl formation by exerting transcriptional control over the ALA synthesizing enzymes (34). It is proposed that BA rapidly and specifically initiate the synthesis of ALA synthesizing enzymes and mimicking the light effect on ALA formation

(42). Consequently, it is postulated that light and cytokinin may induce an increase in cytoplasmic Ca^{2+}. And the major consequence of such increase in cytoplasmic Ca^{2+} is the activation of protein kinases after binding to calmodulin, and leading to regulation of Chl biosynthesis through protein phosphorylations. Therefore, it is probable that phosphorylation of ALA synthesizing enzymes and their transcriptional factors, and also the chloroplast envelope proteins for transportation processes will lead to the regulatory effects of BA and the cations on greening process.

It is believed that further investigations on the light and cytokinin regulation of gene expressions for ALA synthesizing enzymes, as well as isolation and characterization of ALA synthesizing enzymes from cucumber cotyledons is a requisite for revelation of the elusive pathways that lie between light, cytokinin and greening process.

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